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Top figure: Internalized TRAIL in a LX2 hepatic stellate cell
Bottom figure: Schematic depiction of TRAIL signaling in the liver

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Contents

Addressing liver fibrosis by tumor necrosis factor -related apoptosis-inducing ligand (TRAIL) targeted to hepatic stellate cells

Progressive liver fibrosis is the result of liver injury and is characterized by the excessive accumulation of extracellular matrices. When the progression of chronic liver disease remains unabated, the end-stage disease called liver cirrhosis develops, resulting in a total loss of liver function. Cirrhotic liver is associated with high mortality. As yet, no alternative preventive actions other than removing the underlying mechanisms of chronic liver disease have been introduced for curing liver fibrosis.

Various underlying disease conditions can promote liver fibrosis, but activated hepatic stellate cells are known to play a central role in the progression of the disease. The changes in gene expression pattern in hepatic stellate cells (HSC) during the activation process enable targeting these genes or their corresponding products as one successful approach in order to modulate HSC function and to inhibit the fibrotic process. Viral- or non-viral- mediated gene delivery of curative genes such as matrix metalloproteinases or proteins that interfere with signaling pathways of pro-fibrotic cytokines, i.e. soluble PDGFR, have previously been shown to slow the fibrotic process. However, due to the complex and ubiquitous nature of fibrosis such an approach does not allow for a full recovery. The elimination of activated hepatic stellate cells is a crucial step in the process of a natural resolution

and reversion of liver fibrosis. Previously, it has been shown that genes associated with inducing dead signals, such as TRAIL receptors, are upregulated on activated HSCs. Earlier studies have shown that the increase in TRAIL receptors on the surface of HSCs during activation is associated with an increase in HSC susceptibility to the TRAIL apoptosis effect. However, the short half-life of TRAIL *in vivo* and the development of anti-apoptotic signaling mechanisms that cause TRAIL resistance in activated HSCs have proven to be a major hurdle in enabling the therapeutic application of TRAIL as an option in treating liver fibrosis. This thesis deals with application of targeting TRAIL genes and proteins as a novel technology having the potential to successfully treat liver fibrosis. In fact, this is the very first study to demonstrate the potential application of TRAIL fusion protein and receptor specific TRAIL variants in targeting with dual functional applications that can selectively eliminate activated HSC and its pro-fibrotic function in the fibrotic liver.

Chapter 1

Application of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) for the treatment of liver fibrosis

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Abstract

Liver fibrosis is the result of an excessive production and over-accumulation of extracellular matrices in the liver. The development of liver fibrosis is associated with progressive chronic liver disease. The underlying pathophysiology of the disease presents hepatic stellate cells (HSCs) transitioning into an active form as the major event in the development of fibrosis. Therefore, a desirable anti-fibrotic therapy to eliminate activated HSC is considered the first step toward resolution of liver fibrosis. TRAIL has already been introduced as a selective agent that can induce apoptosis in activated HSC. However, the dual role of apoptosis in progression and resolution of liver fibrosis presents a real dilemma. Uncovering the specific effects of TRAIL on hepatic stellate cells, defining the interaction of TRAIL with different cell populations and characterizing the potential determinants in response to TRAIL during liver fibrosis will not only enable a more comprehensive insight into the role of TRAIL in liver fibrosis but also promote the discovery of safer and more effective therapeutic strategies in using TRAIL for resolving liver fibrosis. This review summarizes the significant findings that contribute to a better understanding of the therapeutic role of TRAIL with regard to liver fibrosis progression and resolution.

Introduction

Following chronic injury, the liver develops the pathology known as fibrosis. The key element in the development of liver fibrosis is a cell type called the Hepatic Stellate cell (HSC). Quiescent HSCs are dedicated to retinoid storage, yet through an activation process following injury they proliferate and transform into a fibroblastic phenotype. In this form, activated HSCs secrete extracellular matrix proteins, mainly collagen I and III, that accumulate over time and affect liver structure and function [1]. Liver fibrosis is considered a dynamic process. A range of growth factors and cytokines, including PDGF, TGF-β and IL-1b, mediate HSCs activation and their sustained proliferation. After activation, HSCs also express tissue inhibitors of metalloproteinase (TIMPs) that block membrane metalloproteinase 1 (MMP-1), a crucial enzyme that degrades and remodels deposited collagen in fibrotic tissue. Inhibition of the production of these cytokines could halt HSC proliferation and promote its phenotypic reversion to a quiescent form. Consequently, an upregulation of MMPs results in the degradation and removal of deposited collagens. However, the chronic nature of liver fibrosis, the underlying diseases and the complex and overlapping profibrotic signaling limit efficient removal of the involved profibrotic elements. Of several mechanisms that promote the resolution of liver fibrosis, the elimination of activated HSC, because of its central role in the fibrotic process, is considered a major step. Indeed, dissecting the mechanisms and pathophysiology underlying liver fibrosis provides a wealth of evidence that associates fibrosis resolution with the apoptotic elimination of activated HSCs. Activated HSCs are especially prone to the apoptotic agonist TRAIL as a result of higher expression of

its dedicated receptors on their surface [2,3]. In recent years, research has been conducted regarding the potential application of TRAIL as a tumoricidal agent. However, less attention has been paid to its use as an anti-fibrotic element. In this review, we discuss the benefits and deficiencies of TRAIL as an anti-fibrotic agent in liver fibrosis.

TNF-related ligands

TNF-α, CD59L (Fas L) and TRAIL are among the most studied factors in the TNF family that induce apoptosis and thus cell death. These ligands are naturally employed by a number of immune cells, especially NK and CTL cells, to induce controlled apoptosis in tumor cells or infected cells However, depending on the modulation and signaling pathway that they initiate and develop, their corresponding targeted cells undergo different and even contradictory consequences [4]. HSC cells have receptors for all three types of the mentioned ligands. CD59 induces cell death in activated HSC through JNK-assisted tyrosine phosphorylation of CD59, while it blocks the apoptotic pathway via the CD95 receptor tyrosine nitration and even has a thriving effect on quiescent HSC via the epidermal growth factor receptor (EGFR) phosphorylation [5]. TNF is secreted from mononuclear cells and damaged hepatic cells and binds to TNF-receptor-1 or 2 (TNFR1, 2). TNFR2 may induce cell death in active HSC via the Fas-Associated protein Death Domain (FADD), while TNFR1 signals proliferation and activation in HSCs [6]. TRAIL is mainly produced by activated natural killer cells or macrophages in a membrane-anchored or soluble form [7]. NK cells are particularly important in eliminating activated HSCs in liver

cells. For this reason, the liver accommodates NKCD56^{Bright} cells that have been specialized to produce substantial amounts of TRAIL ligands in comparison with peripheral NKCD56^{dim} cells [8]. NK cells from Hepatitis C Virus-infected patients efficiently eliminate primary activated HSCs *in vitro* in a TRAIL dependent manner [9]. Also, treatment with IFN-Y and IFN-α upregulates TRAIL expression of TRAIL- expressing NK cells in the liver and makes a significant contribution to viral clearance and the resolution of liver fibrosis [10–12]. On the other hand, cytokines such as TGF-β and IL-10 that are produced during chronic liver damage could impair hepatic NK cell function and subsequently its efficiency in resolving fibrosis [13–15]. Two types of apoptosis-inducing TRAIL receptors have been identified: TRAIL-R1 (also referred to as DR4) and TRAIL-R2 (also called DR5/killer/TRICK2). DR5 is expressed to a higher extent on the surface of activated HSC in comparison to DR4. TRAIL is associated with the induction of cell death through the intrinsic caspase pathway, caspase 9 activation and a distinct TRAIL mediated apoptosis pathway called paraptosis. Paraptosis is an osmotic dysregulation of HSCs induced by prolonged potassium channel activation [12, 16, 17]. In addition to efficient induction of apoptosis, TRAIL does not seem to have the extreme liver toxicity, including massive hemorrhagic necrosis, which is associated with other death-inducing ligands such as CD95 ligand and TNF-α. This makes TRAIL an attractive pro-apoptotic receptor ligand [18]. A direct relation has been established between fibrogenesis and the number of activated HSCs [19]. By removing activated HSCs as the source of extracellular matrix (ECM) production, TRAIL could indirectly down regulate proliferation of remaining HSCs via down

regulation of collagen and TGF-β production as an important activator of HSC [20]. TRAIL is capable of directly inhibiting the production of collagen by HSCs without killing them, because TRAIL does not interfere with the folding mechanism of collagen production. TRAIL regulates collagen production through interfering with nuclear translocation of heat shock factor 1(HsF1). Blocking HsF1 translocation leads to a decrease in Heat shock protein 47 (Hsp47) expression a of collagen-specific molecular chaperones in activated HSCs that are responsible for the correct folding and secretion of pre-collagen to form soluble collagen[21].

Selectivity of TRAIL variants in elimination of activated HSCs

TRAIL has a multivalent affinity for its decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), in addition to the DR4 and DR5 receptors that induce apoptosis. The dynamics of wild type and mutant TRAIL interaction with their receptors has been well characterized [22]. Receptor-specific agonistic TRAIL has been introduced both in the form of receptor-specific mutant TRAIL agonists or monoclonal antibodies against specific TRAIL receptors such as DR4 and DR5 [23–25]. Since the DR5 receptor is over expressed up to 105-times in activated HSC in comparison to quiescent HSCs [26], using DR5 agonists could be a highly efficient therapy for specific elimination of activated HSCs. DR5-specific agonists have been shown to reduce the decoy receptor-mediated antagonism more than 20-times. Thus, using DR5 receptor specific TRAIL should lower the required administered dose, possibly with fewer side effects [22, 27]. The application of receptor-specific agonists for DR5 could drastically reduce [24] the hepatotoxicity that is associated

with the use of wild type human TRAIL [23–25, 28]. This toxicity could be due to negligible amount of DR5 in comparison to DR4 on the surface of healthy hepatic cells [24, 28]. In some pathological cases, such as nonalcoholic steatohepatitis, steatotic hepatocytes release toxic saturated free fatty acids which induce the expression of DR5 and initiate receptor localization into cell surface lipid rafts with subsequent recruitment of the initiator caspase-8 upon binding to TRAIL [29].

Adverse effect of TRAIL on healthy liver hepatic parenchyma

A point of discussion in using TRAIL related agonists in clinical treatment has mainly centered on its unwanted effects on normal tissues, especially hepatic parenchyma. TRAIL is known to induce apoptosis in various organs: thymocytes, prostate epithelial cells and neural cells under certain conditions [28, 30–32]. In addition, some studies indicate TRAIL treatment is a source of potential damage to normal hepatic parenchyma [33]. However, hepatic cell death *in vivo* was substantially decreased in TRAIL-deficient mice or in the presence of TRAIL receptor inhibitors [34]. As stated above, apoptosis and the state of liver fibrosis are linked**.** Receptor-mediated apoptosis of liver cells initiates the release of chemotactic signaling mediators including macrophage inflammatory protein-2 (MIP2), monocyte chemotactic protein-1 (MCP-1) and CXC ligand-1 that recruit macrophages into the liver and promote hepatic inflammation. The engulfing of Apoptotic Bodies (ABs) by quiescent HSC facilitates the transformation to the fibroblastic phenotype of activated HSCs and the release of fibrogenic mediators including TGF-β. TGF-β increases apoptosis in hepatic cells as well as the

expression of ECM by HSCs (Figure 1) [17,35,36]**.** Linking TRAIL with other molecules could increase the formation of different TRAIL conformations and develop toxic responses toward hepatic cells [31, 37–40]. Moreover, the hepatic models that are used to evaluate the effects of TRAIL on normal hepatic parenchyma are inconsistent. Freshly isolated human but not nonhuman primate hepatocytes were found to be sensitive to TRAIL apoptosis [37–40]. Further studies, however, have shown that this was an *in vitro* artifact caused by the isolation procedure and the adaptation to culture conditions [38,39]**.**

TRAIL and increased hepatotoxicity in viral hepatitis

The state of viral hepatitis seems to be related to TRAIL-induced apoptosis. However, there is still a controversy regarding the exact role of TRAIL in viral induced hepatitis. Different viral components modulate the cellular response towards TRAIL. *In vivo* models for adenoviral hepatitis showed that apoptosis in infected hepatocytes was mediated by down regulation of the TRAIL decoy receptor [34]. Adenovirus E1A gene expression increases the cellular susceptibility towards TRAIL-induced apoptosis. On the other hand, E1B and to a greater extent E3 10.4K and 14.5K proteins (also known as E3-RID) neutralize the effect of TRAIL in affected cells[41]. The E1B 19K protein inhibits the activation of procaspase-8 (FLICE) through FLICE sequestration, thus rendering infected cells resistant to TRAIL apoptosis, while E3-RID facilitates the internalization and degradation of TRAIL receptor 1(DR1) [41]. However, most other types of viral hepatitis make hepatic cells vulnerable to the effects of TRAIL [42]. Hepatitis B Virus and its HBX

protein sensitize hepatocytes to TRAIL-induced apoptosis through up regulation of Bax protein [42], while the hepatitis C virus sensitizes the infected cells through up regulation of TRAIL receptors [43]. HIV glycoprotein gp120 binding to CXCR4 chemokine receptor selectively up-regulates TRAIL R2 expression on hepatocytes through JNK 2 kinase and confers an acquired sensitivity to TRAIL mediated apoptosis. Interestingly, co-infection of HCV and HIV increases hepatocyte apoptosis in comparison to HCV or HIV alone. It is supposed that this effect is mediated by the simultaneous TRAIL receptor and ligand up-regulation [44, 45].

Role of Cytokines in TRAIL-mediated resolution of liver fibrosis

TRAIL expression in the liver is highly inducible by a number of cytokines, including interleukin-2 (IL-2), interferon gamma (IFN-γ) and Interferon α and β (IFN α, β). IL-2 increases TRAIL expression in liver NK cells, whereas this effect is not present in peripheral NK cells [8]. Liver NK cells are important eliminators of liver fibrosis and, upon activation by IL-2, liver NK cells enhance TRAIL expression to induce apoptosis in their target cells [12]. IFN-γ is another cytokine that has a proven effect on reducing liver fibrosis. Several mechanisms have been proposed for the anti-fibrotic effect of IFN-γ in liver fibrosis, including interfering with TGF-β signaling, inhibiting HSC activation, slowing activated HSC proliferation and consequently reducing extracellular matrix secretion and deposition [46–49]. However, recent findings support the role of IFN-γ in enhancing bound and soluble TRAIL expression by NK-T cells and NK cells that further boost the killing efficiency of these effector cells against activated HSCs [7, 8, 12, 50].

IFN-γ also down regulates the TRAIL-Rs in healthy hepatic cells and therefore reduces the collateral damage to these cells [43, 44, 50, 51]. Both IFN- α and IFN-β antagonize the TGF-β signaling and SMAD3 stimulated collagen transcription in activated HSCs [52, 53]. IFN- α / β also induce TRAIL expression of NK cells through activation of the IFN stimulated gene factor-3 (ISGF3) transcription factor and play a critical role in limiting fibrosis during viral hepatitis[54]. In fact, clinical studies indicate that long term administration of IFN- α / β alleviates non-established liver fibrosis and reduces extra cellular matrix levels [53, 55].

HSCs sensitivity to TRAIL-mediated apoptosis and synergy with drugs

Activated HSCs are in constant need of supporting survival signals in order to sustain their proliferation and fibrotic state. Still, removal of fibrogenic cells is considered the first natural step toward resolving liver fibrosis. There is a delicate balance between apoptotic removal of activated HSCs and ABs in promoting fibrosis. Phagocytosis of the ABs by activated HSCs delivers survival signals to HSCs and promotes the progression of fibrosis. This process is mainly regulated through two pathways; sensing ABs reminiscence by the toll like receptor 9 on HSCs initiates a MYD88-dependent pathway of Nuclear factor kappa B (NF-κB) activation[35]; or, alternatively, of the phosphoinositide 3 kinase (PI3K)-dependent phosphorylation of NF-κB and subsequent translocation of NF-κB into the nucleus. Incorrect regulation of NF-κB has been linked to many inflammatory diseases, including liver fibrosis. NF-κB is responsible for inducing the expression of survival genes, including the anti-apoptotic Bcl-2 family proteins Bcl-XL. A direct

relation has been established between the amount of contra-apoptotic proto-oncogenes Bcl-XL in different stages of HSC activation and susceptibility toward death-inducing ligands [56]. The inhibition of NF-κB could therefore offer a potent mechanism for the direct induction of apoptosis in activated HSCs or for their sensitization to apoptosis by TRAIL agonists. Sulfasalazine and related compounds are commonly used as anti-inflammatory drugs that activate IκB kinase (IKK), an intermediate that activates the inhibitor of nuclear factor kappa-B subunit beta (I-Kβ) and that accelerates the recovery from liver fibrosis by eliminating activated HSCs [57]. Proteasome inhibition could prevent I-kB degradation, and thus inhibition of NF-κB. This effect is associated with the loss of survival proteins and consequent cell death. Inhibitors of the proteasome such as Bortezomib and MG132 induce apoptosis in activated and immortalized human HSCs. In addition, this inhibition elevates DR5 expression on the surface of HSCs and hence renders them more susceptible to TRAIL agonists [58]. Interestingly, TRAIL itself may induce NF-κB via TRAIL-induced activation of the JNK pathway in HSCs cells [59]. JNK pathway activation has been associated with a range of effects, including collagen expression and cell proliferation in HSCs. Leflunomide, a JNK inhibitory compound, prevents cell proliferation and subsequently enhances TRAIL- mediated apoptosis in culture-activated HSCs [59].

Role of growth factors in TRAIL mediated apoptosis

ECM producing cells such as HSC are highly responsive to a series of growth factors, such as EGF, Amphiregulin (AR), Beta Cellulin (BTC) and Platelet Derived

Growth Factor (PDGF), due to the increased expression of the corresponding receptors on their surfaces [60-62]. Upon attachment to their dedicated receptors, growth factors such as PDGF and EGF form homo or hetero dimmers and induce signal transduction through the cytoplasmic tail of the receptor. These factors play a pivotal role in the development of liver fibrosis because they increase HSC proliferation through a series of signaling kinases, including extracellular Kinas 1⁄2 (ERK1/2), Focal Adhesion Kinase (FAK), Phosphoinositide 3-kinase /Protein Kinase B (PI-3K/Akt) and c-Jun N-terminal kinase 26 (JNK 26). These growth factors may also block TRAIL-mediated apoptosis by interfering with the caspase-3 p17 phosphorylation [5, 60, 63]. The EGF family represents transmembrane anchored proteins on the surface of hepatocytes and HSCs. However, upon activation, HSCs increase the amount of free EGF in the environment through a process called ectodomain shedding. Ectodomain shedding occurs due to higher expression of membrane metalloproteinase, such as ADAM 12 in activated HSCs. ADAM 12 proteolytically cleaves and releases EGF-like ligands anchored on the surface of HSC in the liver environment [64]. Interestingly, TRAIL itself accelerates ectodomain shedding of EGF, hence antagonizing its apoptotic effect [63]. Targeted therapy of activated HSCs via an anti-EGF receptor scFv and a TRAIL fused protein was shown to be more efficient in eliminating activated HSC viability and ECM secretion [65].

TRAIL signaling: a balance between inducing apoptosis and killing fibrosis

Liver cirrhosis is considered an end stage liver disease and is the primary cause of the need for liver transplant. The development of liver fibrosis is associated with progressive chronic liver diseases. The best anti-fibrotic therapy is elimination of the underlying disease process. In situations where treating the underlying etiology is not possible or not sufficient for reversing the process, specific anti-fibrotic therapy would be highly desirable. HSCs play central role in liver fibrogenesis [2, 3]. Activation of HSCs is associated with the overexpression of death-inducing receptors such as TRAIL, which makes activated HSCs ideal targets for inducing apoptosis through TRAIL agonists. However, the safe and efficient application of TRAIL for the resolution of liver fibrosis requires addressing several issues. Due to the flow of survival- and growth factors that are released during the fibrotic process, efficient induction of apoptosis requires the circumvention or blocking of these signals. An even greater concern is TRAIL-induced hepatotoxicity and the notorious effect of TRAIL on parenchymal hepatic cells as innocent bystanders. Cytokines, inflammatory factors, viruses and fatty acids render hepatic cells susceptible to TRAIL, a consequence not observed in healthy conditions. In addition, the bifunctional nature of apoptosis as the nexus of liver injury and fibrosis casts doubt over the benefit of this approach. It is thus of crucial importance to direct the effect of TRAIL toward the activated HSCs while avoiding collateral damage to hepatic parenchyma. Such pinpoint accuracy might be achievable through a few customized strategies. Application of receptor-specific TRAIL or TRAIL fused with receptor-

specific ligands could circumvent binding to decoy receptors and induce more efficient apoptosis in activated HSCs. Simultaneous application of TRAIL and other inhibitors of inflammatory mediators of signaling and interferons could sensitize HSCs to TRAIL while protecting hepatocytes against the lethal effects of TRAIL. Finally, recent advances in gene delivery could enable the sustained local production of TRAIL in the liver via customized vectors, which would enable a limited distribution and a more efficient therapeutic administration of TRAIL in fibrosis-affected aerials while avoiding systematic effects of TRAIL [65].

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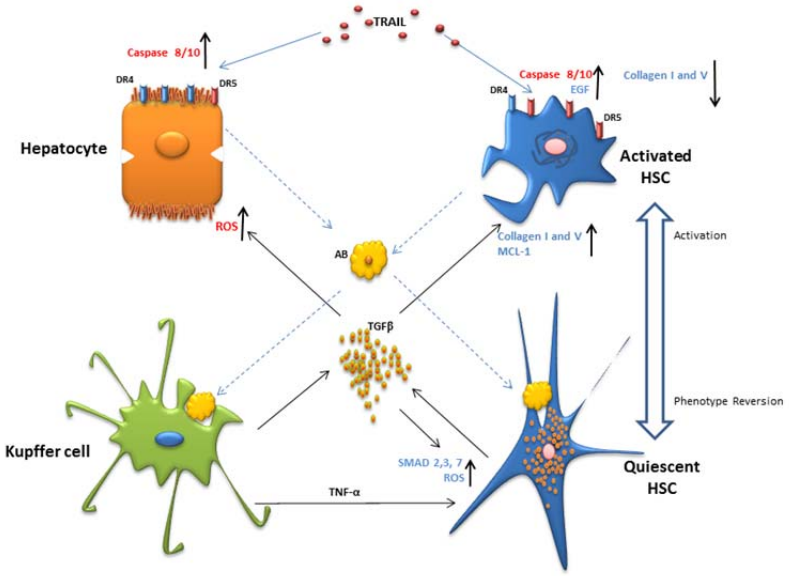

Figure 1: Relation between TRAIL-induced apoptosis and different cell types communication with emphasis on the central role of AB and TGF- β. The direct effect of TRAIL on activated HSCs is considered to be ameliorating liver fibrosis via elimination of these cells.. The indirect effect of TRAIL increases inflammation and accelerates fibrosis. MCL 1; *Myeloid Cell Leukemia 1 protein*, ROS; *Reactive oxygen species*, EGF; *Epidermal Growth Factor*

Chapter 2

Targeted elimination of activated hepatic stellate cells by an anti-epidermal growth factor-receptor single chain fragment variable antibody-tumor necrosis factor-related apoptosis-inducing ligand (scFv425-sTRAIL)

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Abstract

Background Progressive liver fibrosis is the result of chronic liver injury and is characterized by the excessive accumulation of extracellular matrix that may result in liver failure. Activated hepatic stellate cells are known to play a central role in this process and their elimination is a crucial step towards the resolution and reversion of liver fibrosis. In the present study, we investigated the potential application of an anti-epidermal growth factor receptor single chain fragment variable antibody-tumor necrosis factor-related apoptosis inducing ligand (scFv425-sTRAIL) fusion protein in the targeted elimination of activated hepatic stellate cells.

Methods Activated hepatic stellate cells (LX2 cells) were treated by adenovirus-derived scFv425-sTRAIL to evaluate its effect on the viability and extracellular matrix production.

Results *In vitro* treatment of activated hepatic stellate cells with scFv425-sTRAIL induced a significant reduction in viability (up to 100% reduction) and extracellular matrix production (60% reduction), yet no significant effect was observed on hepatic parenchymal cells. Blockage of the epidermal growth factor receptor (EGFR) by a monoclonal antibody significantly reduced the effectiveness of scFv425-sTRAIL in activated hepatic stellate cells, whereas a reduced effectivity was also observed after inhibition of the caspase pathway.

Conclusions Evidence is presented for the successful application of the scFv425-sTRAIL fusion protein in the targeted elimination of activated hepatic stellate cells via EGFR and simultaneous activation of the caspase pathway. scFv425-sTRAIL may thus represent a new therapeutic compound against liver fibrosis.

Keywords EGFR; liver fibrosis; targeted therapy; TRAIL

Introduction

Following chronic injury, the liver may develop into a pathologic state referred to as fibrosis. The key factor in this process is the hepatic stellate cell (HSC). Although the HSC is dedicated as a retinoid storage cell in its original quiescent form, upon activation, it starts the secretion of extracellular matrix (ECM), mainly collagens I and III, which may accumulate over time and affect normal liver functions [1]. Despite the advances that have been made with the aim of understanding the molecular mechanisms and the pathophysiology underlying liver fibrosis, an effective therapeutic approach still remains elusive. The central role of HSCs during liver fibrogenesis makes this cell type an ideal target to stop and even reverse liver fibrosis[2,3]. To achieve this, several conventional drugs such as transforming growth factor (TGF-β) inhibitors, as well as more targeted unconventional methods, such as gene therapy [2,4][5], have been introduced and brought some progress to the field. Induction of apoptosis in HSC as the nexus of liver fibrosis gave also this approach major attention [3,6].

Although some drugs such as IDN-6556A, gliotoxin and sulfasalazine induced apoptosis and elimination of HSCs *in vitro*, the lack of overall efficiency and the cytotoxicity associated with these treatments form a barrier for its use in the clinic

[7,8]. Activation of HSCs is associated with the overexpression of death-inducing receptors such as tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors, including DR4 and DR5, and hence render HSCs susceptible to the apoptotic effects of TRAIL agonists [6,9]. The application of TRAIL agonists has thus been described as a potential strategy to eliminate activated HSCs [9]. However, survival factors such as epidermal growth factor (EGF) may interfere in this process by blocking the downstream pathway of apoptosis and supporting the growth and proliferation of active HSC. Interestingly, it was shown that the presence of TRAIL ligands in itself can cause the shedding of growth actors that adds to the protection and survival of active HSC [10]. On the other hand, the ubiquitous expression of TRAIL receptors and the complex role of apoptosis in inducing inflammation make it even more difficult to explore TRAIL ligands for the treatment of liver fibrosis .[3,6] A problem that is usually associated with inefficient TRAIL treatment is over responsiveness of activated HSCs to growth factors including EGF [11–13] that could amplify activated HSC proliferation and protect against TRAIL apoptosis by interfering with downstream caspase cascade signaling. In the present study, we demonstrate an increase of EGF receptor (EGFR) expression associated with progressive activation of HSCs. EGF ligands are found in free and membrane bound forms. During the activation process, membrane metalloproteinases could release membrane bound EGFs into the environment through a process called shedding. Interestingly TRAIL molecules itself can also up-regulate the EGF shedding and contribute to TRAIL resistance. We previously showed that a dual function targeted fusion protein could suppress the function of

the EGFR at the same time as inducing ligand-assisted apoptosis[14,15]. This fusion protein is well characterized and size exclusion chromatography data showed that eukaryotic expressed single chain fragment variable (scFv)-TRAIL fusion proteins are present as active thermostable homotrimers, with no detectable inactive monomers or dimers present [16]and could successfully eradicate tumor cells carrying both EGF and TRAIL receptors [14,15]. Employment of an adenovector as a carrier for transferring therapeutic genes has already been explored for a numbers of gene therapy applications. An interesting feature of this vector is its inherent property for homing to hepatic cells through its specific so called coxsackie adeno receptor. Our approach for employing adenovirally produced 425-TRAIL is particularly beneficial because TRAIL has very short half-life (30 min) and needs high dosages and frequencies of administration to be able to show clinical effect [16,17].Therefore, we consider that targeted, stable and in situ production of scFv425-TRAIL should result in the *in vitro* elimination of activated HSCs and a reduction of collagen production. This property could lead to more efficient targeting and further elimination of active HSCs. Therefore, we assessed the effect of an adenovirally expressed soluble TRAIL ligand-anti-EGFR antibody (scFv425) fusion protein for its potential therapeutic application in the targeted resolution of liver fibrosis. A schematic representation of the proposed active mechanism of scFv425-sTRAIL is shown in Figure 1. The results obtained in the present study show that the scFv425-sTRAIL bi-functional fusion protein efficiently eliminates *in vitro* activated HSC and reduces collagen production and α-smooth muscle actin (α-SMA) expression in treated cells.

Materials and methods

Cell line and culture

LX-2 Immortalized human hepatic stellate cell line were kindly provided by Prof. Scott Friedman (Mount Sinai Hospital, New York, NY, USA) and were cultured in Dulbecco's minimum essential medium (DMEM; Glutamax, Gibco, Gaithersburg, MD, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 g/ml streptomycin, 50 g/ml gentamicin and 100 nmol/l insulin. HepG-2 human hepatoma cells (ATCC#HB-8065), Huh-7 human hepatoma cells and HEK-293 human embryonic kidney cells (ATCC# CRL-1573) were cultured in DMEM containing 10% fetal bovine serum.

Antibodies and inhibitors

The following antibodies (Ab) were used: anti-TRAIL monoclonal Ab anti-Human CD253 (TRAIL) (eBioscience Affymetrics Co., Carlsbad, CA, USA), mouse anti-EGFR immunoglobulin (Ig)G2a, Mouse anti-α-SMA IgG, Goat anti-collagen-I IgG Ab (Sigma, St Louis, MO, USA), anti-hemagglutinin (HA) mouse IgG1 (InvivoGen, San Diego, CA, USA) anti-ß-actin mouse IgG (Sigma). For inhibition of the caspase pathway the pan-caspase inhibitor FAMVAD-fmk carboxyfluoresceinVAD-fmk (Bachem, Bubendorf, Switzerland) was used.

Production of adenovirally expressed scFv425-sTRAIL and TRAIL

scFv425-sTRAIL was produced using transduction of 293 T cells with a multiplicity of infection (MOI) of 5 from the Ad easy1-scFv425-sTRAIL recombinant vector and harvested 72 h later as described previously [15]. The supernatants containing the fusion protein were screened using western blotting with an anti-HA Ab to check

for expression of the 53-kDa fused protein. For evaluation of efficient trimerization of the adenovirally produced scFv425-TRAIL a western blot was performed under non-denaturing conditions (Figure 2). Ad-sTRAIL was generated by introducing the gene encoding sTRAIL into E1- and E3-deleted replication-incompetent recombinant Ad-5 adenovirus using the Ad-Easy system (Agilent Technologies Inc., Santa Clara, CA, USA). In short, polymerase chain reaction (PCR) amplification of sTRAIL gene from scFv425-sTRAIL included adenovirus [14]using forward primer (AGGCCCAGCCGGCCACCTCTGAGGAAACCAT) and reverse primer for SV40 poly adenylation signal (GAAATTTGTGATGCTATTGC) and DNA was inserted using SfiI and EcoRV unique sites of pAdTRACK-CMVscFv425: sTRAIL [15]The resulting vector was co-transformed with the adenoviral genome vector pAdEasy1 in Escherichia coli BJ5183[18]. After homologous recombination, pAd-sTRAIL was obtained. Subsequently, pAd-sTRAIL was transfected into HEK-293 cells. The cell lysate and supernatant containing the protein were screened
using western blotting with an anti-HA Ab to check for the expression of the 26-kDa protein. The control virus AdTL is an E1- and E3-deleted recombinant serotype 5 adenovirus that contains a green fluorescent protein (GFP) and luciferase gene expression cassette, each under the control of a cytomegalovirus promoter, has been described previously [19].

Evaluation of scFv425-sTRAIL effect on activated LX2 cell viability and proliferation

To activate the LX2 cells, cells (1000 per well) were seeded and incubated in 96-well, flat-bottomed uncoated plastic plates in DMEM, Glutamax (Invitrogen)media with 1% fetal bovine serum (FBS) for up to 7 days as described previously [9]. Cells

were exposed to various concentrations of scFv425-sTRAIL or sTRAIL diluted in DMEM medium for 48 h. Cell only controls received DMEM 1% FBS and virus controls received different concentrations of control adenovector (AdTL). The effect of different constructs and agents on cell viability was assessed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate) in accordance with the manufacturer's instructions (Sigma) in triplicate. For evaluating the effect of caspase inhibition on preventing the apoptosis induced by scFv425-sTRAIL, commercially supplied lyophilized FAM-VAD-fmk pan caspase inhibitor was reconstituted to a final concentration 2 μg/ml in DMEM. The media of activated LX2 cells were replaced by FAM-VAD-fmk DMEM 24 h prior to adding 1.6 nM of adenovirally expressed scFv425-sTRAIL fusion protein. Induced apoptosis was evaluated by measuring the viability after 48 h by the MTS assay. For evaluation of the role of EGFR in scFv425-sTRAIL apoptosis, 1 μg/ml of anti-EGFR IgG2a Ab was added 24 h prior to adding scFv425-sTRAIL fusion protein to block the EGFR on the cells.

Western blotting

For SDS-PAGE and western blotting, LX2 cells were cultured in T75 flasks at 50% confluency. TGF-ß (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to medium at a concentration of 2 ng/ml and, after 48 h, scFv425-sTRAIL was added to a final concentration a 0.3 nM or 1.6 nM. For control cells, only DMEM was added to activated LX2 cells. Forty-eight hours later, the supernatants were removed and cells were washed with phosphate-buffered saline (PBS). Some 500 μl of Laemli buffer was added and the samples were separated on 12.5% polyacrylamide

gels. For natural view of protein trimerization proteins are prepared in a non-reducing non-denaturing sample buffer, without adding 2- mercaptoethanol and boiling. Gels were stained with Coomassie Brilliant Blue R250 for protein visualization. For western blot analysis with Ab against collagen I, α-SMA and β-actin, gels were blotted on activated polyvinylidene fluoride membranes with electrophoretic transfer overnight at 4 °C in blotting buffer. The membrane was then blocked for 1.5 h with 3% bovine serum albumin (BSA)–0.05% Tween in PBS. It was then incubated for 2 h with an Ab against human collagen I diluted 1 : 1000, α-SMA diluted 1 : 1000 or β-actin diluted 1 : 5000 separately in PBS with 1% BSA and 0.05% Tween. After washing three times for 5 min with PBS–0.05% tween, the membrane was incubated for 1 h with the second Ab [rabbit anti-mouse IgG, horseradish peroxidase (HRP) conjugated (Dako, Glostrup, Denmark) for β-actin and α-SMA and mouse anti-goat IgG-HRP (Dako) in the case of collagen I], all diluted 1 : 1000 in the buffer described above. The membrane was then washed four times for 15min in the washing solution used above. The blot was developed using an AEC staining solution system (Sigma) in accordance with the manufacturer's instructions.

Immunohistochemistry

For immunohistochemistry of cultured activated LX2 cells, first, LX 2 cells were cultured on uncoated plastic 96-well plates with 1% FBS in DMEM for 7 days, and were then treated with different amounts of scFv425-s TRAIL. After 48 h, the medium was removed and the cells were washed three times with PBS, fixed with absolute methanol (Sigma) at –20 °C for 20min, air dried for 30 min and re-hydrated

with PBS for 5min. Next, the cells were incubated with anti-collagen I diluted 1:75 or anti-α-SMA diluted 1:600 in PBS. Finally the cells were washed with PBS three times and incubated with the second Ab [rabbit anti-mouse IgG-HRP (Dako) for α-SMA and mouse-anti Goat IgG-HRP (Dako) for collagen I, all diluted 1:100 in PBS. Finally, cells were washed with PBS three times and stained using the acetylcholinesterase (Sigma) staining solution system in accordance with the manufacturer's instructions.

Fluorescence-activated cell sorting (FACS) analysis of DR4, DR5, DecoyR1 and DecoyR2 expression on quiescent and activated HSCs

For flow cytometer analysis, HSCs were detached using 0.05% Trypsin-EDTA in PBS, washed with ice-cold PBS, and diluted to a concentration of 2×106 cells/ml using cold PBS. Aliquots of 100 µl (2×105 cells) were centrifuged at 18 g. for 5min at 4 °C, the supernatant was discarded, and the cells were suspended in either 100 µl of rabbit anti-DR4 (10µg/ml), rabbit anti-DR5 (10 µg/ml) rabbit anti-DecoyR1 (10 µg/ml) or rabbit anti-DecoyR2 (10 µg/ml) antibodies for TRAIL receptors and anti-human EGFR (5 µg/ml). After incubation for 45min at 4 °C, the cells were washed twice with PBS and were incubated for an additional 45 min with 1:100 diluted anti-rabbit PE-conjugated secondary antibody under cold and dark conditions. After two final washings, cells were suspended in 300 µl of PBS (1% BSA). Unlabeled cells and cells labeled with secondary antibody alone served as negative controls. The mean values of fluorescence intensity of 10 000 cells were determined by FACS analysis (Calibur 1; Becton-Dickinson Biosciences, Franklin Lakes, NJ, USA) and analyzed with FlowJo VX software (Tree Star Inc., Ashland, OR, USA).

Caspase 3/7 assay

Caspase 3/7 activities were assayed by using Apo-ONE™ Homogeneous Caspase-3/7 Assay kit (Promega. Madison, WI, USA). Briefly, 2×104 cells from 425-TRAIL, TRAIL treated quiescent and activated HSCs, 2×104 control cells and blank (no cells) were transferred into a 96-well plate and 100 µl of Homogeneous Caspase-3/7 Reagent was added. The plate was covered with a plate seal. After incubation for 48 h, fluorescence of each well was measured at an excitation wavelength of 485nm and an emission wavelength of 530 nm.

RNA isolation and real-time reverse transcriptase (RT)-PCR analysis

Total RNA from cultured LX2 cells was isolated using the SV total RNA isolation system (Promega Z3100) (all tests carried out in triplicate) in accordance with the manufacturer's instructions. The amount of Runaways measured by Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, USA) and analyzed qualitatively by gel electrophoresis. Subsequently, synthesis of first-strand cDNA from total RNA was performed with the Reverse Transcription System (Promega A3500) in a volume of 20µl containing 250 ng of oligo dT (Promega). The cDNA obtained was diluted with Millipore water (Millipore Corporation, Billerica, MA, USA) to a concentration of 10 ng/µl and1µlwas applied for each PCR reaction. α-SMA primer was ordered as Assays by- Design (Applied Biosystems, Foster City, CA, USA) (4331348/assay name ACT-R-ACT2). GAPDH was used as a housekeeping gene (Rodent GAPDH Control Reagent; Applied Biosystems). The PCR reaction was carried out in TaqMan PCR Master Mix (Applied Biosystems) with a final concentration of 200 nM for GAPDH primers and 250 nM for primers of

the other genes under investigation. The amplification reaction was performed in an ABI PRISM 7900HT sequence detector (Applied Biosystems) with the cycling conditions: 2 min at 50 °C, 10min at 95 °C and 40 two-step cycles of 15 s at 95 °C and 60 s at 60 °C. For each sample, the real-time PCR reaction was performed in triplicate, and the averages of the obtained threshold cycle (Ct) values were processed for further calculations in accordance with the comparative Ct method described in the ABI manual (http://www.appliedbiosystems.com). In brief, gene expression levels were normalized to the expression of the housekeeping gene GAPDH, giving the ΔCt value. Then, the average value of ΔCt obtained from day 0 culture LX2 was subtracted from the average of the ΔCt value of each sample, yielding the ΔΔCt value. Finally, the gene expression level was calculated as $2^{-\Delta\Delta Ct}$, giving the final value that is normalized to the housekeeping gene and relative to the control sample values of the studied α-SMA gene.

Protein quantification

Quantitation of protein expression in western blots was carried out by scanning the blots using a GS-710 calibrated imaging densitometer (Bio-Rad, Hercules, CA, USA) and Image J, version 1. 46 (NIH, Bethesda, MD, USA) by putting a frame around the desired band and calculating the occupied area for each band in comparison with the standard band.

Results

Progressive activation of LX2 cells in cell culture

To characterize the activation stat of LX2 cells, we cultured cells for 9 days on plastic. This model of progressive activation in culture has already been established and well characterized for LX2 cells for *in vitro* study of liver fibrosis [9].LX2 cells were harvested and screened for signs of activation via mRNA expression of α-SMA, an indicative marker of activation, during different time points. An increase of five-fold in mRNA expression of α-SMA was associated with the progressive culture (Figure 3). This model was further used to evaluate the effect of different TRAIL constructs on activated HSCs.

scFv425-sTRAIL reduces viability of activated LX2 cells

To evaluate the effect of adenovirally produced scFv425- sTRAIL, different amounts of supernatant containing scFv425-sTRAIL or sTRAIL were added to activated LX2 stellate cells and the viability of cells was determined 48 h later by the MTS assay. Activated LX2 cells showed a reduction of viability of up to 90% after treatment with scFv 425-sTRAIL at a concentration of 0.6 nM compared to only 50% inhibition by sTRAIL alone (Figure 4A). The inhibition of viability in quiescent HSC was 60% after treatment with 1.6 nM scFv425-sTRAIL and 20% with 1.6 nM sTRAIL (Figure 4B). Thus, the EGFR-targeted TRAIL shows a relative selectivity towards activated stellate cells. To exclude the role of adenovirus transduction in decreased proliferation of LX2 cells both activated and quiescent LX2 cells were transduced by a MOI of 50 of a control GFP-expressing adenovirus. The results showed no effect on the viability of LX2 cells (data not shown). Finally, to examine the specificity of scFv425-sTRAIL, hepatic parenchymal cells (huh7 and Hep-G2) were treated with scFv425-sTRAIL. Both cell lines showed no detectable

difference in viability after application of scFv425-sTRAIL (Figure 4C), confirming the selectivity of the fusion protein towards EGFR expressing cells.

scFv425-sTRAIL employs both EGFR signaling and the apoptosis pathway

To evaluate the contributions of the EGFR and apoptosis pathways, we used selective inhibitors during the treatment of LX2 cells with scFv425-sTRAIL fusion protein. Activated LX2 cells were exposed to scFv425-sTRAIL and an EGFR specific Ab or a caspase inhibitor. At a concentration where scFv425-sTRAIL induced no survival at all, significant viability (30%±4%) was observed in the presence of the anti-EGFR Ab (Figure 5). Similarly, inhibiting the caspase cascade by addition of the global caspase inhibitor (FAM-VAD-fmk) was associated with a significant inhibition of the effect of scFv425-sTRAIL (20%±5% viability). These results suggest that the 425-sTRAIL fusion protein reduced viability in activated stellate cells by blocking both the EGFR and caspase-associated apoptotic pathways. An increase in susceptibility to TRAIL is associated with the expression of TRAIL-R1/DR4 and TRAIL-R2/DR5 receptors, yet the resistance to TRAIL is considered partially a result of the presence of decoy receptors TRAIL-R3/DcR1 and TRAILQ8R4/DcR2 therefore we assessed the expression of these receptors using FACS analysis (Figure 6). In accordance with the observed activation profile for α-SMA expression in activated HSCs, there was increased DR4 and DR5 receptor expression. TRAIL-R1/DR4 expression increased by 9.0% and TRAILR2 / DR5 expression increased by 5.6%. However, the rate of TRAIL-R2/DR5 expression in comparison with DR4 was 10-fold higher, which means that DR5 plays the major role in TRAIL-mediated apoptosis. The expression of both TRAIL-R3/DcR1 and

TRAILR4/DcR2 also increased in HSCs after activation, yet TRAILR4/DcR2 increased to a greater extent. EGFR expression increased by 5% on the surface of activated HSCs. We next evaluated the contribution of TRAIL receptor expression with respect to sensitivity towards our TRAIL constructs. Measurement of caspase activity in HSCs treated with scFv425-TRAIL showed an increase of 22% and 26% in caspase3/7 activity in quiescent and activated HSCs compared to TRAIL-treated cells. Caspase activity increased by 60% in activated HSCs treated with scFv425-TRAIL compared to quiescent HSCs treated with scFv425-TRAIL. This is almost two-fold higher than caspase elevation as a result of TRAIL treatment of activated HSCs (Figure 7).

scFv425-sTRAIL reduces collagen I and α-SMA expression

One of the characteristic of activated HSCs is the increased expression of fibrotic markers such as α-SMA and collagen I. To evaluate the potential effect of scFv 425-sTRAIL on α-SMA and collagen I expression, LX2 cells were cultured on a plastic surface for 5 days and different amounts of scFv425-sTRAIL were added to cell cultures. After 48 h, cells were stained with anti-collagen I and anti-α-SMA. We observed a reduction in the expression of α-SMA and collagen in activated LX2 cells in response to a sub-lethal dose of scFv425-sTRAIL (0.32 nM). Quantification of staining indicated a reduction in expression of collagen I and α-SMA of up to 46% and 60%, respectively (Figure 8). Thus, the addition of scFv425-sTRAIL to activated HSCs at a sub-lethal dose could reduce collagen production and HSC activation.

Discussion

Persistent liver injury initiates a cascade of events including secretion of a number of cytokines such as TGF-β, platelet-derived growth factor (PDGF) and endothelin-1. These cytokines activate HSCs and this leads to exponentiation of ECM production and accumulation, a condition referred to as liver fibrosis. HSCs have a central role in this process, which makes them an ideal target in the treatment of liver fibrosis. Although liver fibrosis is a serious situation that may eventually develop into a life-threatening condition, a cure still remains elusive [2,4]. It has been shown that activated HSCs significantly amplify the hepatic response to liver injury, and depleting fibrotic livers from activated HSCs may ameliorate the fibrotic condition [8]. TNF, CD95L (Fas) and TRAIL are among the most studied factors in TNF family members that could induce death and apoptosis in cells. The ligands are employed by a number of immune cells specially natural killer and cytotoxic T lymphocyte cells to induce controlled apoptosis in tumor or infected cells [20]. However, depending on the modulation and the signaling pathway that they initiate, their corresponding target cells can experience different and even contradictory consequences [21]. HSC cells have the receptors for all three types of ligands. CD95L may induce cell death in activated HSCs through JNK-assisted tyrosine phosphorylation of CD95, whereas it could block the apoptotic pathway via CD95 tyrosine nitration and even have a thriving effect on quiescent HSC via EGFR phosphorylation [22]. TNF is secreted from mononuclear cells and damaged hepatic cells and exerts effect via TNF-receptor-1 or 2 (TNFR1 or 2). Upon binding to its dedicated receptor, TNFR2 could induce cell death in active HSC via the Fas-

associated protein death domain, yet, as a result of interaction with TNFR1, it can cause proliferation and activation in HSCs, and hence enhance liver fibrogenesis [23]. TRAIL, either soluble or membrane-attached, is primarily produced by activated natural killer cells and Kupffer cells to induce apoptosis in their target cells. Two types of TRAIL-binding receptors have been identified: TRAIL-R1 (also referred to as DR4) and TRAIL-R2 (also called DR5/killer/TRICK2) that are expressed on the surface of HSCs, However, DR5 is expressed to a higher extent on the surface of the activated HSC and is responsible for the induction of death through the extrinsic caspase pathway and caspase-8- dependent activation [24]. One of the appealing features

of TRAIL as a pro-apoptotic receptor ligand is that it does not appear to have the liver toxicity precluding the testing *in vivo* of related death-inducing ligands such as CD95 ligand and TNF-α, which both cause massive hemorrhagic necrosis of various tissues including the liver [25]. Previously, TRAIL-induced apoptosis was successfully used to eliminate active HSC [9]; however, simultaneous overexpression of EGF-like ligands in fibrotic and cirrhotic livers [11,13] and its downstream signaling and protection of activated HSCs represents a hurdle to achieving effective TRAIL-based treatment [11,22,26]. In the present study, we therefore developed a selective treatment for the elimination of activated HSCs via EGFR targeted TRAIL to enhance the selectivity TRAIL and thus improve the remedy. TRAIL receptors overexpress in activated HSC and are therefore an ideal targets for TRAIL agonists. We showed a reduction in viability of 20% and 50% in quiescent and activated stellate cells, respectively, via exposure to adenovirally

expressed sTRAIL at a concentration 0.6 nM. This finding is in concordance with previous studies that showed sensitivity of HSCs to TRAIL [9]. ECM-producing cells such as HSC are highly responsive to a series of growth factors such as EGF, amphiregulin, β cellulin and PDGFand their dedicated receptors which may trigger survival and/or proliferation signaling through the EGFR and/or PDGFR on their surface [11–13]. Indeed, growth factors such as amphiregulin have been proven to have a pivotal role in the development of liver fibrosis via different mechanisms; involving extracellular Kinas½(ERK1/2), transcription factor c-fos and TRAIL [12]. Meanwhile, the contribution of TRAIL ligands in accelerating ectodomain shedding of EGF and heterodimer activation of PDGF could add to the complex protection layer and survival of activated HSCs [10] . An enhanced apoptosis may be achieved by combining the TRAIL treatment with compounds that block the protective mechanisms, giving reason for us to couple trail with anti-EGFR antibodies. This has the additional benefits of directing soluble TRAIL to EGFR-expressing cells, that is, activated HSC. Indeed our previous studies have already confirmed the potent anti-tumor [23,25]effect of such a compound in mouse models with EGFR-expressing tumors [14,15]. In the present study, we show that an adenovirus derived fusion protein scFv425-sTRAIL can induce a 100% viability loss in activated versus 50% in quiescent HSCs. This finding is in accordance with the elevation in caspase 3/7 signaling in affected HSCs. Blocking the EGFR on HSCs through a mouse monoclonal Ab to eliminate the EGFR signaling pathway reduced the efficiency of scFv425-sTRAIL and increased viability by up to 30%. This finding highlights the importance of a simultaneous approach for coupling death signaling and survival

inhibition for eliminating activated HSCs. On the other hand, inhibition of the caspase cascade via a global inhibitor also attenuated the effect of scFv425-sTRAIL indicating that both caspase-based and EGFR-based mechanism are involved in the apoptosis induction by the fusion protein. An interesting observation in the present study is the role of scFv425-sTRAIL in reducing collagen I expression in activated HSC. Collagen I is a major component of ECM. Inhibition of EGF receptor signaling [27]may result in the attenuation of fibrosis reduction via inhibition of collagen deposition and HSC activation) (Figure 4) [17], next to the induction of apoptosis. A direct relation has already been established between a reduction fibrogenesis and the number of α-SMA positive cells [4]. A reduction in the amount of α-SMA has been demonstrated in the presence of some inhibitors of EGFR signaling, such as Gefitinib and Erlotinib. Treatment of pulmonary artery smooth muscle cells with these drugs reduced α-SMA expression by 10% [28]. Because the reduction in fibrilar collagens in the present study was accompanied by a reduction in the number of α-SMA positive cells, it may be speculated that decreased expression of ECM proteins is caused by a reduction in HSC activation. The later could be attributed to inhibitory effect of trimeric form of anti-EGFR in the fusion protein against EGFR in activated HSC [27]. Because fibrosis is considered as rather a long-term and persistent problem, a durable and efficient approach is needed if an objective and non-causative cure is to be pursued. Our current strategy involves treating activated HSCs with an adenovirally produced scFv425-sTRAIL protein. The next step is the in vivo production of this fusion protein by adenovirus-transfected hepatocyte cells. The natural homing property of adenovirus towards the

liver and the HSC-specific nature of the expressed molecule add to the specificity of our designed strategy. The present study is the first to show a significant anti-fibrotic effect of this compound in HSC, which warrants further studies in vivo.

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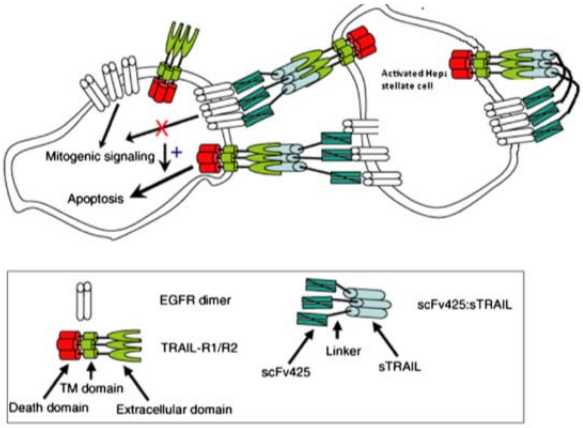

Figure 1. Schematic representation of the fusion protein and its cellular interactions. Binding of the anti-EGFR antibody to activated HSCs in the fusion protein (scFv425-sTRAIL) causes inhibition of cell proliferation and provides a scaffold that facilitates the binding of TRAIL to its receptor on HSC in Cis and Trans form.

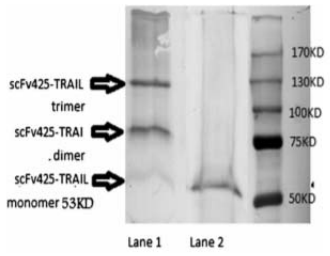

Figure 2. scFv 425-TRAIL trimerization western blot analysis of adenovirally expressed scFv425-TRAIL under nondenaturing (lane 1) and reducing (lane 2) conditions showed efficient trimerization of the scFv425-TRAIL fusion construct under non-reducing conditions.

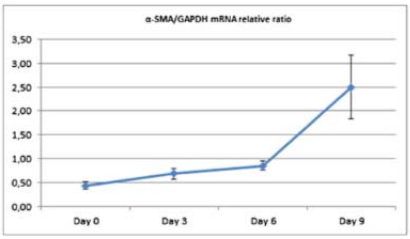

Figure 3. ALpha-SMA mRNA expression increases following replating on a plastic surface. Human LX2 cells were grown to confluence and then lifted and replaced on the plastic surface of six-well plates for 9 days. At day 9, cells were harvested. α-SMA mRNA was extracted and quantified by real-time RT-PCR as described in the Materials and methods. An increase in α-SMA mRNA is associated with the length of time in culture.

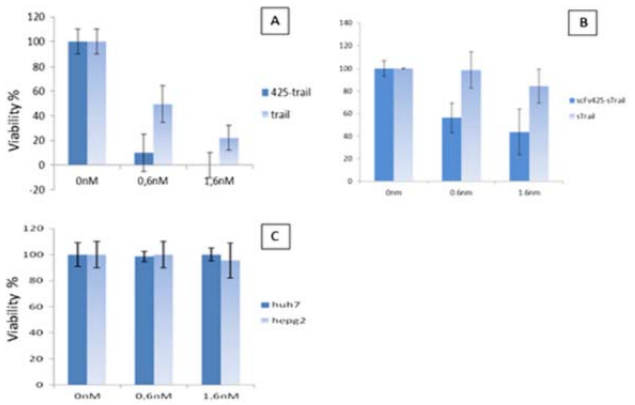

Figure 4. scFv425-sTRAIL efficiently kills activated HSCs. (A) MTS viability assay for activated LX2 cells. (B) MTS viability assay for quiescent LX2 cells. Dark bar, scFv425-sTRAIL; light bar, sTRAIL. (C) parenchymal hepatic cells (Hep-G2 and Huh7). Dark bar, huh7; light bar, HEPG2. The effects of scFv425-sTRAIL were most prominent in activated LX2 cells and absent in parenchymal hepatic cells.

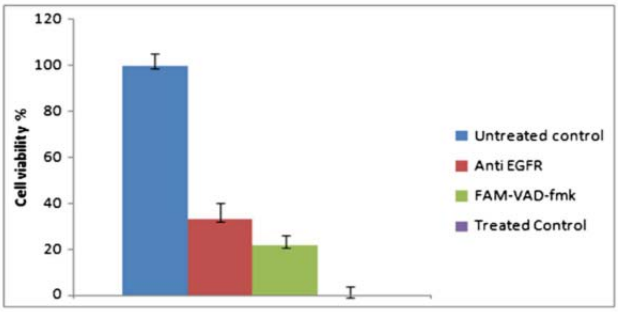

Figure 5. Simultaneous interaction with EGFR and caspase cascade signaling pathways is crucial for efficient killing of active HSC by scFv425-sTRAIL. Activated LX2 cells incubated with Ad scFv425-sTRAIL (violet) or left untreated (blue). scFv425-sTRAIL-treated cells were subsequently treated with 50 μM FAM-VAD-fmk (green) or with 1 μg of anti-EGFR Ab (blue) to block the effects of the caspase cascade and EGFR, respectively. It can be seen that both inhibitors affected the activity of scFv425-sTRAIL.

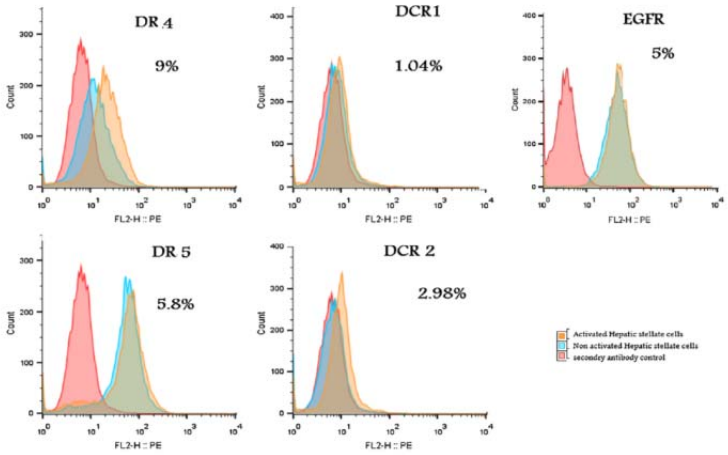

Figure 6. FACS analysis profiling of different TRAIL and EGF receptors and their expression on the surface of quiescent and activated forms of human HSCs. An increase in the expression of surface receptors is associated with the activation of HSCs during culture-induced activation. DR5 is expressed almost ten-fold more than DR4 on the surface of human HSCs. Expression of decoy receptors (DCRs) increases with activation of HSCs; this increase is higher for DCR2.

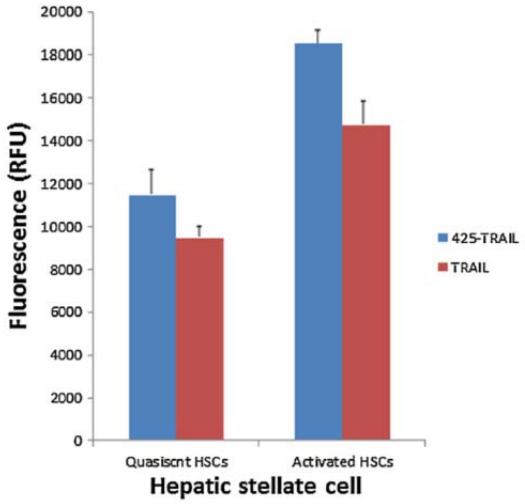

Figure 7. Comparison of caspase 3/7 enzyme activity in quiescent and activated HSCs treated with adenovirally produced 425-sTRAIL or sTRAIL. An increase in the amount of caspase-3 activity in LX2 cells treated with 425-sTRAIL or sTRAIL is concurrent with the activation state of cells. 425-sTRAIL could induce more caspase activation in quiescent and activated hepatic stellate cells compared to sTRAIL.

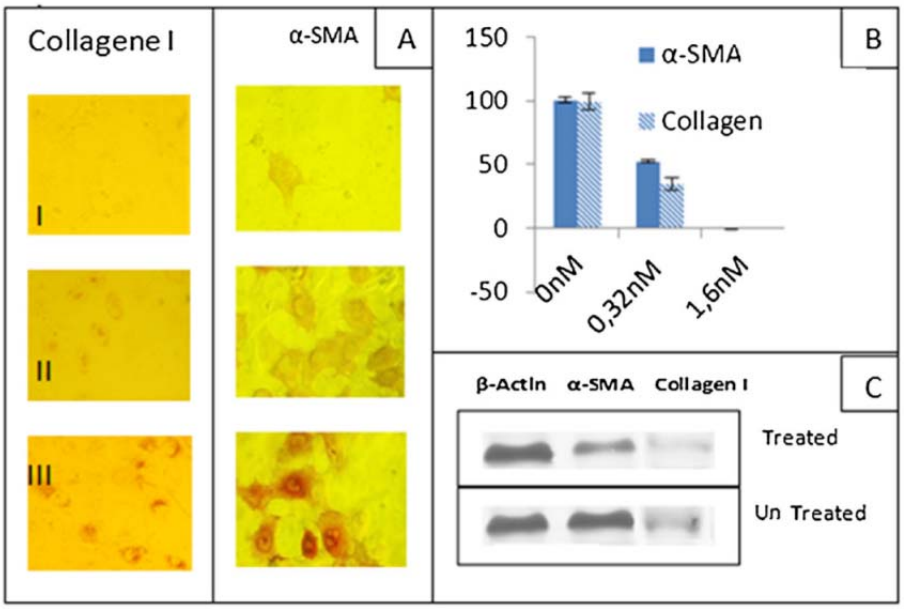

Figure 8. scFv425-sTRAIL reduces the expression of extracellular matrix proteins in activated HSCs. (A) LX2 cells were treated with (I) 1.6 nM, (II) 0.3 nM and (III) 0 nM of scFv425-sTRAIL and stained for collagen (left) or α-SMA (right). (B) Bar graph of collagen (light bar) and α-SMA (dark bar) expression as determined by western blotting before and after treatment with 0.32 nM scFv425- sTRAIL. (C).Representative western blot staining for β-actin, α-SMA and collagen I of cultures of activated LX2 cells with (upper) and without 0.32 nM scFv425-sTRAIL (lower).

Chapter 3

Targeted elimination of activated hepatic stellate cells by PDGF-TRAIL and EGF-TRAIL (receptor-specific ligand peptide – sTRAIL) fusion proteins

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Abstract

Background Activation and proliferation of HSCs during chronic liver injury cause progressive replacement of hepatic parenchyma with fibrotic tissue; whereas apoptosis appears as a significant event to reduce the number of activated HSCs during the resolution phase of hepatic fibrosis. In nature, Tumor necrosis factor-Related Apoptosis-Inducing Ligand (TRAIL) has the major role in this dynamic process by inducing apoptosis in activated HSCs. Therefore, application of TRAIL is considered a desirable anti-fibrotic therapy. However, the very short half-life of TRAIL *in-vivo* and the dual role of apoptosis in progression and resolution of liver fibrosis demands strategies to increase the selectivity of TRAIL for HSCs while reducing dosage for maximal effect. The present study addresses this issue by targeting TRAIL to activated HSCs via PDGF or EGF receptor specific peptide moieties.

Methods Activated HSCs (LX2 cells) were treated by PDGF receptor specific peptide (pPB) or EGF receptor specific peptide (GE11) moieties linked to the extracellular part of TRAIL in the form of fusion proteins. We then evaluated the viability of affected LX2 cells and extracellular matrix production by the affected LX2 cells.

Results *In vitro* studies showed that treatment of activated HSCs with pPB-TRAIL or GE11-TRAIL did not reduce the viability of activated HSCs to a greater extent than TRAIL alone. Evaluation of TRAIL binding to activated HSCs using confocal microscopy revealed that targeting via anti-EGFR single chain antibody; targeting

via pPB or targeting via GE11 did not increase the binding of TRAIL toward activated HSCs.

Conclusion The application of pPB-TRAIL or GE11-TRAIL fusion proteins did not increase the potency of TRAIL molecule in the targeted elimination of activated HSCs. Our findings indicate that the higher binding capacity of anti-EGFR scFv-TRAIL towards activated HSCs could be accountable for its greater efficiency in eliminating these cells .

Introduction

Gene Therapy using fusion peptide PDGF-sTRAIL and EGF-sTRAIL

Activated HSCs are the major basis cause of liver fibrosis. It has been shown that activated HSCs significantly amplify the hepatic response to liver injury, and depleting fibrotic livers from activated HSCs could ameliorate the fibrotic condition [1]. Therefore, an ideal therapy for liver fibrosis should be able to selectively eliminate activated HSCs in fibrotic liver. We and others have successfully employed TRAIL-receptor agonists to target and eliminate activated HSCs [2,3]. However, the expression of membrane-bound and free TRAIL decoy receptors 1 and 2, that neutralize the effect of TRAIL, impose a hurdle to achieve the efficient application of TRAIL for resolution of liver fibrosis. Moreover, the ubiquitous expression of TRAIL receptors and the complex role of apoptosis in inducing inflammation is a reason to explore more targeted and more efficient TRAIL ligands for the treatment of liver fibrosis [2].

An ideal way to minimize the potential deleterious side effects of TRAIL therapy in liver fibrosis is to channel the apoptotic property of TRAIL specifically to activated HSCs. To address this, a number of moieties may be targeted on the surface of activated HSCs to complement with the effect of TRAIL. Receptors for Platelet Derived Growth Factor (PDGF), Epidermal Growth Factor (EGF) and TGF-β are among the highly expressed receptors on activated HSCs that are suitable for being targeted [2][4-5]. Therefore, monoclonal antibodies (mAbs), single-chain variable fragment (scFv) and receptor specific peptides with high target specificity against these receptors are suitable for targeting application. We have already successfully employed an anti EGFR–TRAIL single chain fusion protein to eliminate activated HSCs [5]. However, antibodies possess a high molecular weight, limited tissue penetration and species-specific recognition that might be disadvantageous for targeting applications if an in vivo model is to be tested. Targeting with peptides may be efficient for a range of cell types (without cross-species barriers in receptor affinity), hence can be applied for targeting the payloads in a range of hosts [6,7]. A small peptide, CSRNLIDC, (pPB) has been successfully employed in a couple of approaches to target therapeutic proteins or vectors to activated HSCs [8][9]. This peptide binds to the PDGF-Rβ, without initiating the signaling cascade [8][9]. The EGFR-specific peptide, YHWYGYTPQNVI, **(GE11)** is a dodecapeptide produced by phage display. Ge11 binds to the EGF receptor (EGFR), yet does not activate the receptor tyrosine kinase activity. The absence of EGFR activation, as well as the specific attachment of to the EGFR confer GE11 with significant advantages for targeting applications [10]. This ligand has been used for targeted delivery of

vectors, lytic peptides and cytotoxic compounds to highly expressing EGFR cancer cells[11,12].

In the present study, we examine the potential application of adenovirally expressed receptor-specific peptide -TRAIL fusion proteins, which may induce apoptosis in activated HSCs. For this purpose, we devised growth factor receptor-specific peptides pPB- or GE11-sTRAIL fusion proteins that specifically target and eliminate activated HSCs.

Material and Methods

Cell lines and cell culture

The cells lines used in this study were NIH/3T3 (mouse fibroblast cell line ATCC® CRL-1658™), HEK293 (Human embryonic Kidney 293 cells, ATCC® CRL-1573™) and LX-2 Immortalized human HSCs (kindly provided by Prof. Scott Friedman,Mount Sinai Hospital, New York, NY, USA). The NIH/3T3 and HEK293 cell lines were grown in Dulbecco's minimum essential medium (DMEM, Gibco) whereas LX2 cells were cultured in Dulbecco's minimum essential medium (DMEM; Glutamax, Gibco, Gaithersburg, MD, USA). Media were supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 ug/ml streptomycin.

Antibodies

The following antibodies (Ab) were used: Mouse anti-EGFR IgG2a, anti-hemagglutinin (HA) mouse IgG1 (InvivoGen, San Diego , California), Anti-mouse IgG polyclonal antibody (Sigma).

Construction of shuttle vector pCMV-TRACK pPB-sTRAIL and pCMV-TRACK GE11-sTRAIL

The vector pCMV-Track-scFv425-sTRAIL [5,13] was used for the construction of a vector, so-called pCMV-Track-pPB-sTRAIL. To construct the new vector DNA encoding pPB was synthesized and amplified via PCR using forward and reverse primers, 5'GGGAGATCTTCCACCATGGAGACAGACACA3' and 5'ACAATCGATGAGGTTCCGCGAGCAACCAGTGGAACC3', respectively. The restriction site for BglII was introduced in the forward primer and for ClaI in the reverse primer. The digested PCR product was introduced into BglII and ClaI-digested vector, and ligated. The resulting construct was designated pCMV-Track-HA-PDGF-sTRAIL. Colony PCR was performed on the transformed PCR to screen for the right size of the insert via primers 5'-(GGGAGATCTTCCACCATGGAGACAGACACA)-3' and 5'-(GAAATTTGTGATGCTATTGC)-3'. For the expression of the EGFR-targeting fusion protein GE11-sTRAIL, a genetic construct was made using the vector pCMV-TRACK-PDGF-sTRAIL as the starting material. Partially complementary (in bold) oligonucleotides 5'-(GGG AGA TCT TCC ACC ATG GAG ACA GAC ACA CTC CTG CTA TGG GTA CTG CTC TG**G GTT CCA GGT TCC**)-3' and 5'-(CCC GCGGCCGC TAT CAC GTT CTG GGG TGT GTA ACC GTA CCA ATG ATA ACC AGT **GGA ACC TGG AACC**)-3' were used to generate the IgK (leader peptide)-GE11 peptide. After digestion the construct was inserted into the pCMV-TRACK-PDGF-sTRAIL vector to generate the final construct pCMV-TRACK-GE11-sTRAIL. The integrity of the cloning procedure was verified by

digestion reactions using the restriction enzymes ClaI, KpnI, PmeI and SalI. Colony PCR was performed on the transformed PCR to evaluate the right size of the insert via primers 5'-(GGGAGATCTTCCACCATGGAGACAGACACA)-3' and 5'-(GAAATTTGTGATGCTATTGC)-3'.

Production of Adenovirally expressed Ad-pPB-sTRAIL and Ad-GE11-sTRAIL

Ad-pPB sTRAIL and Ad-GE11-sTRAIL were generated using the plasmids encoding GE11-sTRAIL or pPB sTRAIL using the Ad-Easy system. In short, the plasmids were co-transformed with the adenoviral genome vector pAdEasy1 in *Escherichia coli* BJ5183 (2). After homologous recombination, pAd- pPB sTRAIL or pAd-GE11-sTRAIL was obtained. Subsequently, the pAd- pPB sTRAIL or pAd-GE11-sTRAIL plasmids were separately transfected into HEK-293 cells. The cells were incubated for 14 days to produce virus, which was visible as GFP-positive viral plaques. The cell lysates and supernatants containing the proteins were screened using Western Blotting with an anti-HA Ab to check for the expression of the desired proteins. The schematic representation of the pPB-sTRAIL or GE11-sTRAIL protein is shown in figure 1a.

Evaluation of pPB-sTRAIL or GE11-sTRAIL effect on activated LX2 cell viability and proliferation

To activate the LX2 cells, cells (1000/well) were seeded and incubated in 96-well, flat-bottomed uncoated plastic plates in media with 1% FBS for up to 7 days as previously described (3). Cells were exposed to various concentrations of pPB-sTRAIL or GE11-sTRAIL or sTRAIL diluted in DMEM medium for 48 hours. Cell

only controls received DMEM 1% FBS**.** The effects of the different constructs on cell viability was assessed in triplicate using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate (PMS)) according to the manufacturers protocol (Sigma, USA).

Western Blot Analysis

For SDS-PAGE and Western blotting, Hek 293 cells were cultured in T75 flasks at 50% confluency. The different viral construct Ad-HA-pPB-TRAIL, Ad-HA-GE11-TRAIL was added to the medium at a concentration of 10 MOI and after 48 hours cell were harvested using 0.05% Trypsin- EDTA in PBS. For control cells only DMEM was added to Activated HEK293T cells. Cells were re-suspended in 300µl of Laemli buffer and the samples were separated on 12.5 % polyacrylamide gels. Gels were stained with Coomassie Brilliant Blue R250 for protein visualization. For Western Blot analysis with Ab against HA tag, gels were blotted on activated PVDF membranes with electrophoretic transfer overnight at 4˚C in blotting buffer. The membrane was then blocked for 1.5 hours with 3% BSA-0.05% Tween in PBS. It was then incubated for 2 hours with an Ab against mouse anti HA tag 1:1000 in PBS with1% BSA and 0.05% Tween. After washing 3X for five minutes with PBS-0.05% Tween, the membrane was incubated for one hour with the second Ab (Rabbit anti-mouse IgG, horseradish peroxidase (HRP) conjugated; Dako, Denmark) diluted 1:1000 in the buffer mentioned above. The membrane was then washed 4X for 15 minutes in the washing solution used above. The blot was developed using an

AEC staining solution system (Sigma, USA) according to the manufacturer's instructions.

HUMAN TRAIL (CD253) ELISA

An ELISA was performed on the supernatants of cells transduced with the adenovectors bearing the TRAIL constructs. Briefly, T293 Hek cells were cultured in T75 flasks at 50% confluency. Different viral constructs, Ad-HA-pPB-TRAIL, Ad-HA-GE11-TRAIL, Ad-HA-sTRAIL or Ad-HA-425scFv-sTRAIL, were added separately to medium at an MOI of 10 and after 48 hours the supernatants were collected and used in the ELISA. During the first incubation, 100 µl of the standards (3000, 1500, 750, 375, 187.5, 93,75 pg/ml TRAIL), samples or blank (DMEM medium) and a biotinylated monoclonal antibody specific for TRAIL are simultaneously incubated in microtiter plates for 2 hours at room temperature. After washing 3X with washing solution, Streptavidin-HRP was added to the micro titer plates to bind to the biotinylated antibody, incubated for another 2 hours and washed 3X. TMB substrate solution, 100 µl, of was added and incubated to develop a colored reaction product. The intensity of this colored product was directly proportional to the concentration of TRAIL present in the samples and was read in a spectrophotometer at 450 nm.

Immunohistochemistry and confocal microscopy

For immunohistochemistry of cultured activated LX2 cells, LX2 cells were cultured on uncoated plastic slides with 1% FCS in DMEM for 5 days. At day 6, cells were treated with an MOI of 10 of control adenovirus, ADTL (Ad5 expressing

EGFP) in DMEM 10%, at day 7 the media were removed and replaced by medium containing 1 μM of HA-scFv425-sTRAIL, HA-pPB-sTRAIL or HA-sTRAIL at intervals of 0, 5 and 15 minutes. After 15 minutes the cell supernatants were removed and cell were briefly washed with PBS, fixed with formaldehyde 10% (Sigma) at 25ºC for 20 minutes, washed 3 time and incubated with PBS for 5 minutes. Next, the cells were incubated with mouse anti-HA diluted 1:100 and 1/10000 Hoechst 33342 in PBS. The cells were washed with PBS 3-times and incubated with Rabbit anti-mouse IgG diluted 1:100 in PBS. Finally, the cells were washed with PBS for 3-times and mounted. Cells were observed with a confocal microscope, Leica sp8, with excitation wavelength 590nm and emission 617nm for Alexa Fluor 594 and excitation wavelength 360nm and emission 460nm for EGFP and blue laser (360nm) for Hoechst 33342. The Images were processed by Imaris (3D viewer) software.

FACS analysis of HA-scFv425-sTRAIL, HA-pPB-sTRAIL or HA-sTRAIL on activated HSCs

Flow cytometer analysis. HSCs were detached using 0.05% Trypsin- EDTA in PBS, washed with ice-cold PBS, and diluted to a concentration of 2 * 10^6 cells/mL using cold PBS. Aliquots of 100 μL (2 * 10^5 cells) were centrifuged at 14,00 rpm for 5 min at 4ºC, the supernatant was discarded, and the cells were separately suspended in 100 μL of HA-scFv425-sTRAIL, HA-pPB-sTRAIL, HA-GE11-sTRAIL or HA-sTRAIL (60 pM/mL). After incubation for 45 min at 4ºC, the cells were washed twice with cold (4ºC) PBS and incubated for an additional 45 min with 1:100 diluted mouse anti–HA secondary antibody under cold conditions. The cells were washed

with PBS 3X and incubated with the Rabbit anti-mouse IgG-FITS conjugate diluted 1:100 in PBS. Finally cells were washed with PBS 3X and suspended in 300 µL PBS (1% BSA). Unlabeled cells and cells labeled with secondary antibody alone served as negative controls. The mean values of the fluorescence intensity of 10,000 cells was determined by fluorescence-activated cell sorting (FACS) analysis (Calibur 1) and analyzed with FlowJo VX software.

Results

pPB-sTRAIL or GE11-TRAIL were produced successfully in Adenovector (Ad5 system)

The genes encoding the fusion proteins pPB-sTRAIL or GE11-TRAIL were successfully cloned into Ad5. The production and secretion of HA-pPB-TRAIL or HA-GE11-TRAIL was confirmed by western blotting. For both proteins, a 20KD monomer was detected using an anti-HA antibody (Figure 1b). In addition, lower molecular weight breakdown products were detected. To evaluate the efficiency of production and secretion of HA-pPB-sTRAIL or HA-sTRAIL, sTRAIL and scFv425-sTRAIL, an ELISA assay was performed on the supernatants of transduced cells. scFv425-sTRAIL was produced most efficiently from adenovirus-infected HEK cells with a concentration of 60 μg / ml. sTRAIL and PDGF-sTRAIL were produced at much a lower concentration, 8 μg / ml and 6 μg / ml respectively. The expression of GE11-sTRAIL was substantially lower: 1 ug/ml (Figure 1c).

pPB-sTRAIL and GE11-TRAIL reduce viability of activated LX2 cells

To evaluate the growth inhibiting effects of adenovirally produced, HA-pPB-sTRAIL, HA-GE11-sTRAIL or HA-sTRAIL, different amounts of supernatants containing the proteins were added to activated or quiescent LX2 cells and the viability of cells was determined 48 hours later by MTS assay. Activated LX2 cells showed a 63% reduction of viability after treatment with HA-pPB-TRAIL at a concentration of 408 ng/ml compared to 65% inhibition by sTRAIL alone (Figure

2B). The inhibition of viability in quiescent HSC was 20% after treatment with HA-pPB-TRAIL and 21% at 408 ng/µl of sTRAIL (Figure 2A). Since 3T3 (mouse fibroblast) cells are known to be expressing high level of PDGFR on their surface. We then evaluated the effect of adenovirally produced, HA-pPB-TRAIL or HA-TRAIL on this cell. Activated or quiescent 3T3 cells were treated with different amount of HA-pPB-TRAIL or HA-TRAIL and the viability of cells was determined 48 hours later by MTS assay. Activated 3T3 cells showed up to 95% reduction of viability after treatment with HA-pPB-TRAIL at a concentration of 408 ng/ml compared to 86% inhibition by sTRAIL (Figure 2D). The inhibition of viability in quiescent HSC was 15% after treatment with 408 ng/ml of HA-pPB-TRAIL and comparable with sTRAIL at the same concentration (Figure 2C). Thus, sTRAIL and PDGF receptor -targeted TRAIL show a relative selectivity towards activated HSCs and 3T3 cells. However, there was no significant difference between targeting TRAIL via PDGF receptor and via its dedicated receptors in activated or quiescent LX2 or 3T3 cells. Comparison between the HA-GE11-TRAIL and HA-TRAIL effect on activated and quiescent HSCs also showed similar pattern as that of pPB-TRAIL (figure 3A, B).

Comparative attachment and internalization of different TRAIL types

Upon ligand binding, TRAIL receptors are internalized via clathrin-dependent endocytosis and clathrin-independent pathways. An increase in TRAIL internalization could reduce the TRAIL efficiency in initiating the caspase cascade. Interestingly, cell surface-bound TRAIL could better initiate the caspase cascade[14]. We therefore further investigated the effect of TRAIL internalization

on its potential for eliminating activated LX2 cells. pPB-sTRAIL, HA-GE11-sTRAIL, sTRAIL and scFv425-sTRAIL were added to activated LX2 cells in equimolar concentrations. The binding and attachment of the constructs was evaluated using flow cytometry analysis. HA-scFv425-sTRAIL binding to activated LX2 cells was substantially greater (100-times) than that of HA-sTRAIL, HA-GE11-sTRAIL or HA-pPB-sTRAIL (Figure 4A). Interestingly, blocking the EGFR by an anti-EGFR antibody reduced the binding of scFv425-sTRAIL substantially (Figure 4B). Internalization of the scFv425-sTRAIL construct into the activated HSCs was faster in comparison to that of sTRAIL and pPB-sTRAIL proteins. scFv425-sTRAIL was completely internalized after 5 minutes of incubation; whereas sTRAIL and pPB-sTRAIL were completely internalized after 15 minutes. Therefore a more stable anchoring of TRAIL to the cell surface receptors could not be accountable for more efficient induction of apoptosis in activated HSCs.

Discussion

Liver fibrosis is considered as end-stage liver diseases and is the primary reason for liver transplantation. The development of liver fibrosis is associated with progressive liver chronic diseases. To date, many specific anti-fibrotic therapies fail due to lack of overall efficiency and the toxicity. The underlying pathophysiology of liver fibrosis revealed that activated HSCs play a central role, both as a causative and as an effector cell [2,15]. Therefore, research focusing on the targeted elimination of activated HSC as the first step in the resolution of liver fibrosis is highly desired. Activation of HSCs is associated with the overexpression of death-

inducing receptors such as TRAIL. Directing TRAIL to activated HSCs has previously been attempted as a means to eliminate liver fibrosis [3,5]. Despite the successful application of TRAIL to eliminate activated HSCs, the very short *in vivo* half-life of TRAIL and simultaneous over expression of its free and membrane-bound decoy receptors (DcR1 and DcR2) impose a hurdle for the clinical application of TRAIL. For this reason a new strategy that could bring more efficient targeting and long term endogenous expression (e.g., gene delivery) of TRAIL could be desirable.

Activated HSCs are highly responsive to a series of growth factors and cytokines such as EGF, Amphiregulin (AR), Beta Cellulin (BTC), PDGF and TGF-β as a result of the higher expression of their corresponding receptors on these cells [2][4-5][16]. We have previously shown that a dual function anti- human EGFR scFv-sTRAIL fusion protein could target and suppress the function of the EGFR and at the same time induce ligand-assisted apoptosis in activated HSCs in vitro[5]. In the current study, we evaluated the replacement of the single chain antibody with specific binding peptides.

We successfully constructed and produced a fusion protein, Ge11-sTRAIL, by genetically fusing GE11 sequence to the N terminal of soluble TRAIL molecule and expressing the protein from a recombinant Adenovector. A reduction in viability of activated human HSCs and mouse fibroblast was observed after exposure to adenovirally-expressed GE11-sTRAIL. This finding is in concordance with previous studies that showed sensitivity of HSCs to TRAIL constructs [3,5]. Whereas

successful anti EGFR scFv, GE11 targeting could not further increase the effects of sTRAIL in eliminating activated or quiescent LX2[5].

In addition, we used a PDGF receptor targeting peptide. The PDGF-receptors consist of two chains, PDGF-Rα and β, that form dimmers. Whereas, PDGF-Rα is equally expressed on quiescent and activated HSCs, PDGF-Rβ is up-regulated during the transition of quiescent cells into activated HSCs [2]. Therefore, the higher expression of the PDGF-Rβ on activated HSCs make it an ideal targeting moiety [15]. pPB, a peptide that exclusively recognizes and binds to PDGF-Rβ, has been previously employed to direct proteins to a number of cell types including HSC that over express PDGF-Rβ [16–20]. We successfully constructed and produced a fusion protein, pPB-sTRAIL, by genetically fusing pPB sequence to the N terminal of soluble TRAIL molecule and expressing the protein from a recombinant Adenovector.

A reduction in viability of activated human HSCs and 3T3 cells was observed after exposure to adenovirally-expressed pPB-sTRAIL. This finding is in concordance with previous studies that showed sensitivity of HSCs to TRAIL constructs [3,5]. However, pPB targeting could not significantly augment the effects of sTRAIL in eliminating activated or quiescent LX2.

TRAIL-induced apoptosis is associated with the initiation of downstream apoptotic signaling upon attachment to its dedicated receptor. Therefore, binding of TRAIL constructs to targeted cell is the first crucial step that determines the outcome of process. We have shown that fusing a single-chain antibody against EGFR with

sTRAIL boosts the efficiency of TRAIL to induce apoptosis in activated HSCs [4,5]. The comparison between the pPB-sTRAIL, sTRAIL and 425scFv-sTRAIL showed the scFv-fusion protein could substantially increase the binding of TRAIL to the surface of activated HSCs. For being selective a fusion protein should be able to out compete with the natural ligand for its target receptor. Whereas natural TRAL with dissociation avidity constant (K_D), of 2nM, PDGFRβ and EGFR receptors peptide dissociation constant are in the order of 22 nM [10] [19]. Therefore the pPB-TRAIL or GE11-TRAIL fusion proteins might not be able to increase the avidity beyond the wt TRAIL affinity. Upon binding to its dedicated receptors, TNF, CD95 and TRAIL initiate receptor aggregation and recruitment of the adaptor protein such as FADD (Fas-associated death domain) to the cytoplasmic tail of the TRAIL receptor. Recruitment of FADD is associated with conversion of procaspase 8 (the upmost compartment of caspase cascade) to caspase-8 in the context of Death-Inducing Signaling Complex (DISC). Caspase-8 and FADD are not recruited to a CD95 or TNF-induced plasma membrane-bound receptor-signaling complex, but instead, are activated elsewhere within the cell. However, unlike TNF and death ligand CD95, TRAIL internalization is not required for TRAIL-induced apoptosis and it may even dampen the apoptotic property of TRAIL for initiating the caspase cascade. Immobilized TRAIL protein induces more efficient apoptosis in target cells than soluble TRAIL [14]. Therefore, it may be speculated that an increased capability of scFv425-TRAIL in reducing cellular viability could be due to decreased internalization of TRAIL. Examination of TRAIL internalization for different TRAIL constructs by confocal laser microscopy tracking of TRAIL into the late

endosome compartment of cells showed that scFv425-sTRAIL internalizes faster than either sTRAIL or pPB-sTRAIL. Hence, a better effectivity of scFv425-TRAIL construct in comparison with sTRAIL and pPB-sTRAIL could not be due to anchoring of the sTRAIL construct on the surface of cellular membrane via scFv425. The higher binding affinity of scFv-sTRAIL rather than the internalization is relevant for inhibition of HSCs. Finally, binding of the scFv moiety to the EGFR could antagonize the effect of this growth factor and its downstream proliferative and anti-apoptotic signaling. The inhibition of HSCs by the scFv-sTRAIL protein is therefore the combined inhibitory effect of receptor inhibition and apoptosis induction.

In conclusion, our findings indicate that application of pPB-TRAIL or GE11-TRAIL fusion proteins could induce apoptosis in activated HSCs. However targeted elimination of activated HSCs via EGFR or PDGFR specific peptides and simultaneous activation of the caspase pathway did not significantly increase the potency of these molecules compared to wt TRAIL.

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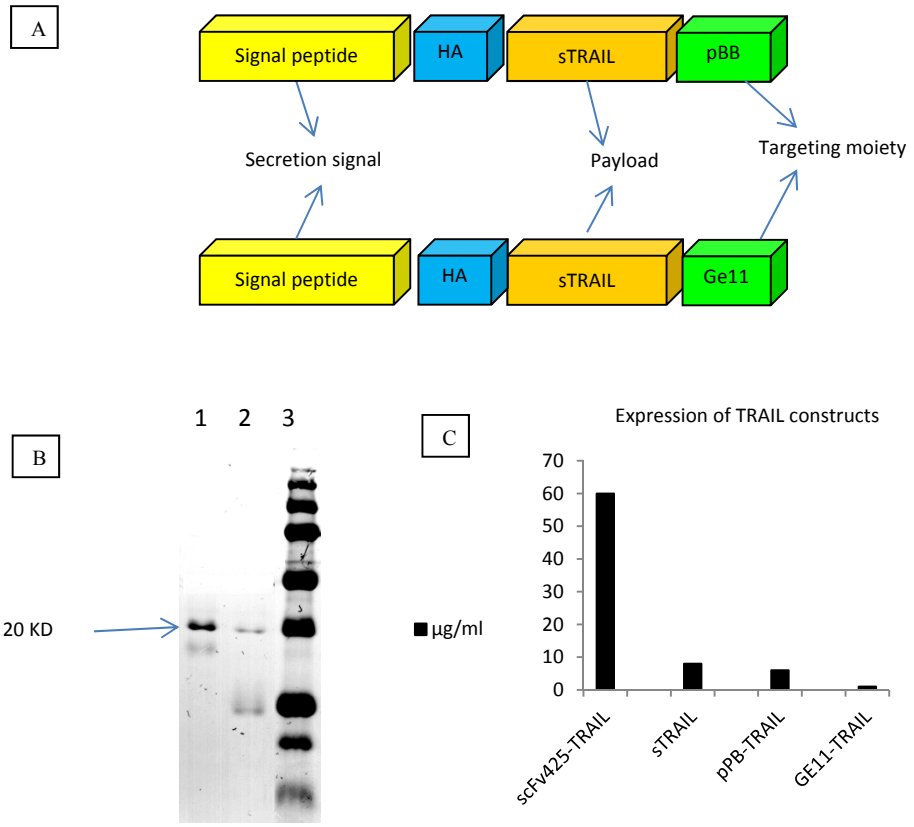


Figure 1: A) Schematic figure of TRAIL-pPB and TRAIL-GE11. B) Western blotting of pPB-TRAIL and GE11-TRAIL showed the band size of 20KD for both proteins lane1: Adenovirally produced PDGF-TRAIL lane 2: Adenovirally produced GE11-TRAIL lane 3: Protein marker. C) Expression of different adenovirally expressed TRAIL constructs by ELISA.

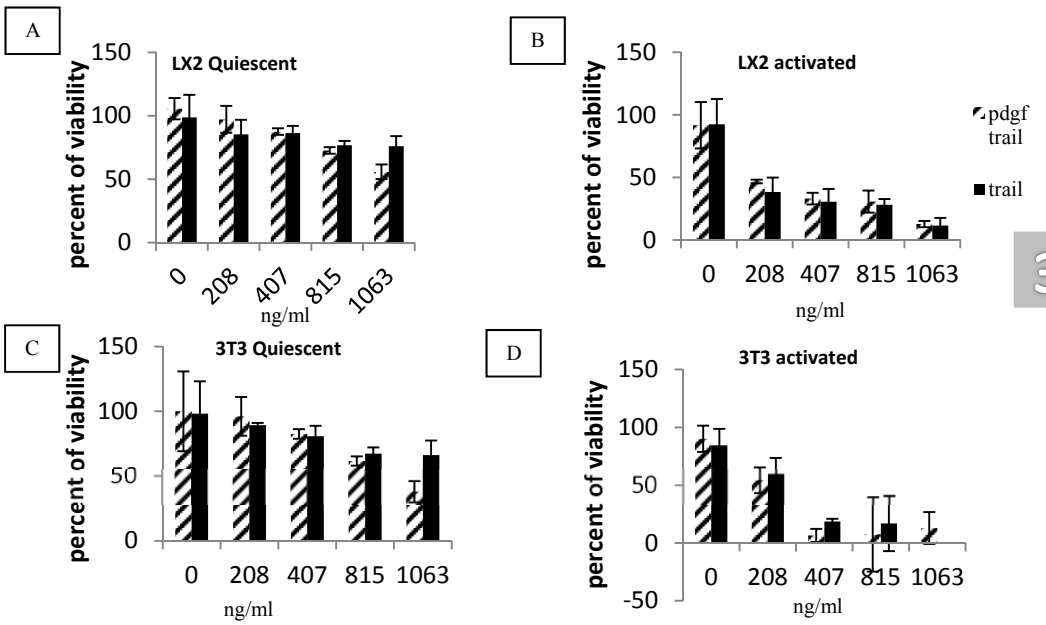


Figure 2: Viability of quiescent (A) and activated (B) LX2 cells treated with different concentration of TRAIL and pPB-TRAIL. Viability of quiescent (C) and activated (D) 3T3 fibroblast cells treated with different concentration of TRAIL and pPB-TRAIL.

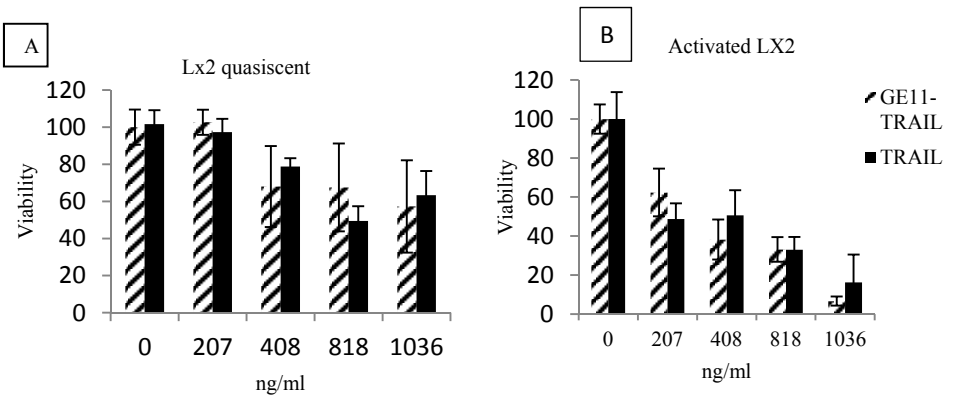


Figure 3: Viability of quiescent (A) and activated (B) LX2 fibroblast cells treated with different concentration of TRAIL and pPB-TRAIL.

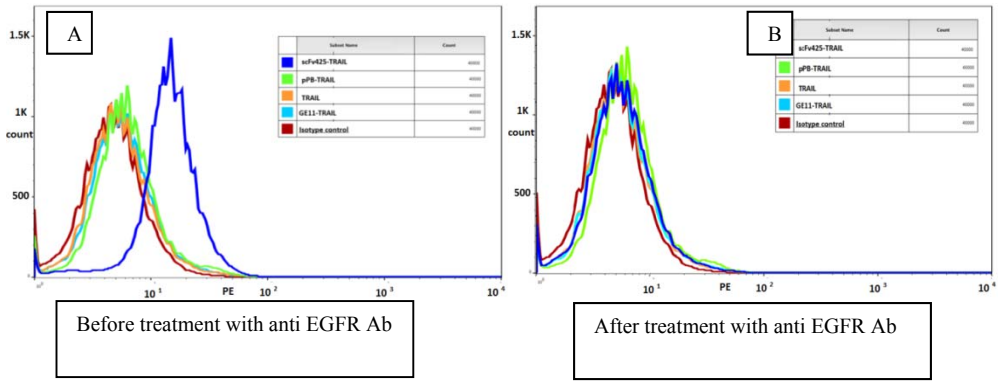
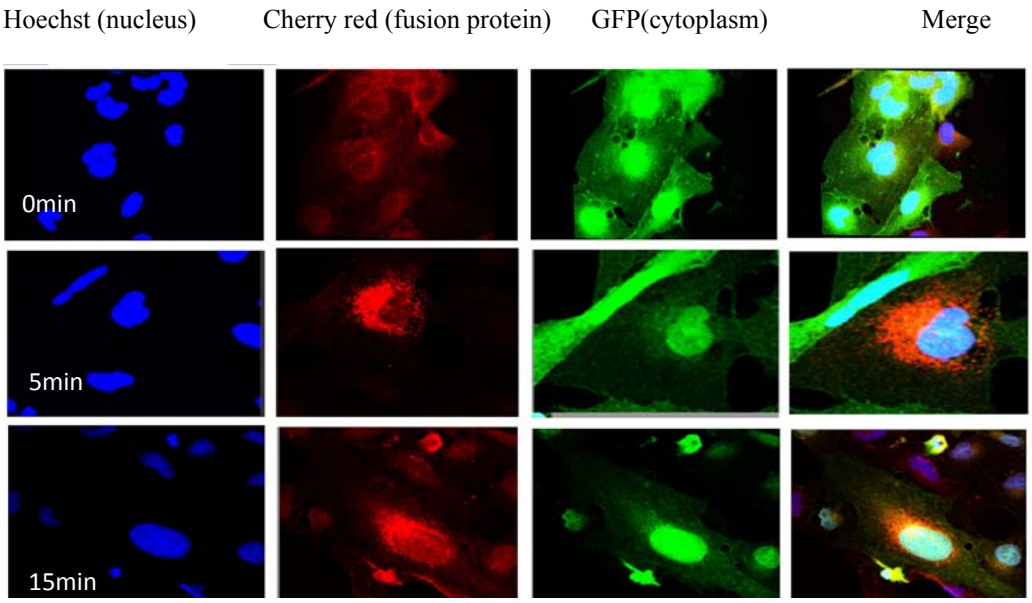
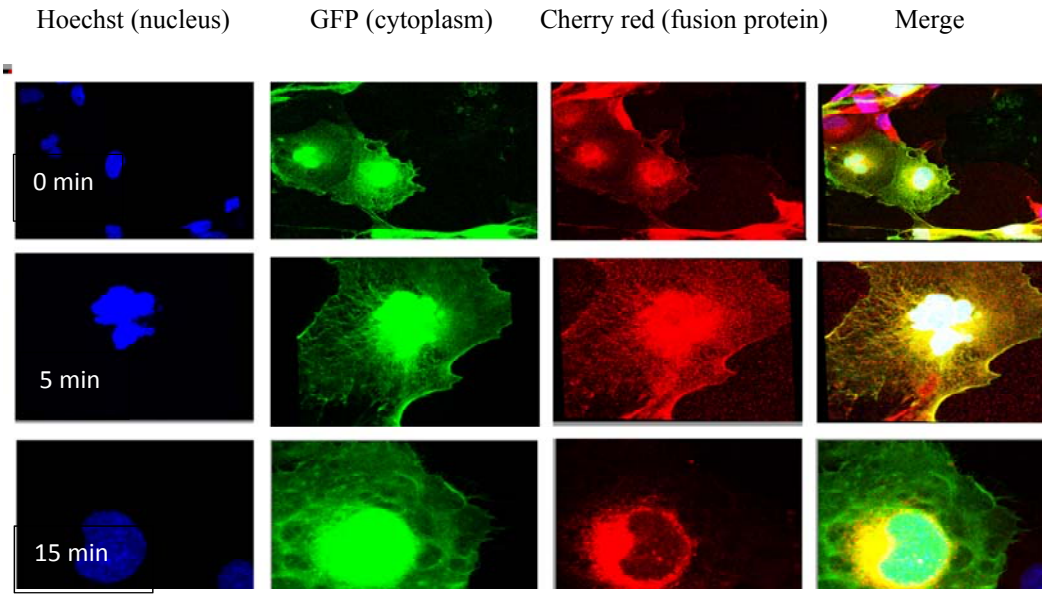


Figure 4: FACS analysis of the binding capacity of TRAIL fusion proteins to activated HSCs (A), Reduction of scFv425-TRAIL binding to LX 2 cells after pretreatment of cells with anti-EGFR (B).

Internalization of TRAIL fusion protein(scFv425-TRAIL) into LX2 cells



Internalization of TRAIL fusion proteins (PDGF-TRAIL) into LX2 cells



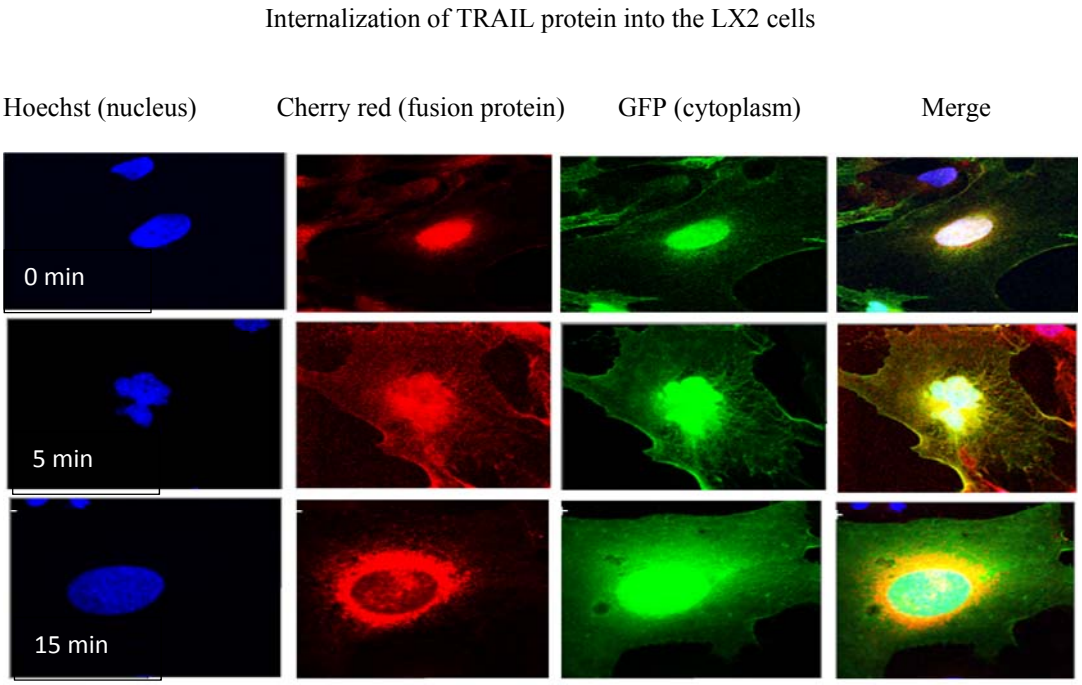


Figure 4: Internalization pattern analysis shows that the internalization of scfv425-TRAIL is faster than that of TRAIL and PDGF-TRAIL. scFv425-TRAIL completely internalized and accumulated around the nucleus after 5 min; whereas TRAIL and PDGF-TRAIL internalized and accumulated around nucleus after 15 min. LX2 cells were transduced with GFP producing Adeasy vectors for 24 hours and incubated with different fusion proteins for different times, fixed with paraformaldehyde and stained for the localization of fusion protein.

Chapter 4

Elimination of activated hepatic stellate cells by receptor-specific TRAIL

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Abstract

Background The activated hepatic stellate cell (HSC) is the major natural target in resolving liver fibrosis. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a molecule that may contribute to the apoptotic removal of activated HSC through binding to its dedicated receptors. In the present study, we investigated the potential application of recombinant receptor-specific TRAIL proteins in the efficient elimination of activated HSCs.

Methods Human LX-2 cell line or rat HSCs were activated by progressive culture on plastic surface. TRAIL receptors expression on the surface of cells were profiled for each specific receptor in activated HSCs. Activated HSCs were treated by receptor-specific TRAIL variants *in vitro* to evaluate its effect on the cell viability and extracellular matrix production.

Results Our finding revealed differential contributions of TRAIL receptors among HSCs populations. Activated HSCs express more TRAIL receptors. Also, HSCs dominantly express DR5 receptors; whereas the side population of HSCs mostly expresses DcR2. *In vitro* treatment of activated HSCs with DR5-specific or wild type TRAIL variants induced a significant reduction in viability and extracellular matrix production. Decrease in viability was associated with treatment of cells only in the presence of high concentration of DR4-specific TRAIL. A decrease in extracellular matrix production was found to correlate with increasing concentration of all TRAIL variants. A pronounced reduction (up to 100%) in collagen production was observed after treatment with DR5-specific TRAIL.

Conclusion our findings provide evidence that more than one receptor system is involved in the recognition and signal transduction of TRAIL into activated HSCs. Evidence is presented for the successful application of the DR5 receptor-specific TRAIL variant in the targeted elimination of activated HSCs via interference with collagen production and simultaneous induction of apoptosis via activation of the caspase pathway. DR5 receptor-specific TRAIL may thus represent a new therapeutic compound for the treatment of liver fibrosis.

Introduction

Following chronic injury, the liver develops to a pathologic state regarded as fibrosis. The key factor in the liver fibrosis process is a cell type called the HSC. Quiescent HSC are dedicated to retinoid storage, yet through an activation process due to injury, they proliferate and transform to a myofibroblastic phenotype. In this form activated HSCs start secretion of extracellular matrix proteins mainly collagen I and III that accumulate over time and impair the functional structure of the liver [1]. During activation, HSC become more susceptible to apoptotic cell death by apoptotic factors, including Tumor Necrosis Factor (TNF)-Related Apoptosis-Inducing Ligand (TRAIL). TNFα, FasL and TRAIL are among the most studied factors in the tumor necrosis factor family members that employed by a number of immune cells, specially natural killer cells(NK) and Cytotoxic Lymphocyte (CTL), to induce controlled apoptosis in tumor cells or infected cells [2]. Whereas TRAIL, CD59L and TNFα may have a proliferative effect on HSCs in certain conditions[3–

5] and could cause massive hemorrhagic necrosis of various tissues, including the liver[6]. Thus TRAIL is the most promising apoptotic ligand to eliminate activated HSCs. There are two death receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2) that are responsible for induction of apoptosis upon binding to TRAIL, yet TRAIL also can bind to the anti-apoptotic decoy receptors DcR1 (TRAIL-R3), DcR2 (TRAIL-R4), and osteoprotegerin (OPG). The application of TRAIL agonists has been suggested as a potential strategy to eliminate activated HSCs[7,8]. On the other hand, the ubiquitous expression of TRAIL receptors and the complex role of apoptosis in inducing inflammation make it difficult to explore TRAIL ligands for the treatment of liver fibrosis[9,10]. TRAIL receptor-specific agonistic molecules have been introduced both in the form of TRAIL variants or monoclonal antibodies against specific TRAIL receptors[11,12]. The dynamics of wild type (wt) and mutant TRAIL interactions with TRAIL death receptors have been well characterized[13]. Receptor-specific agonists may reduce the decoy receptor-mediated antagonism[14], hence by using receptor-specific TRAIL variants the therapeutic dose is expected to be lower[15,16]. The application of receptor-specific agonists for DR5 drastically reduced hepatotoxicity [17] that is associated with wild type human TRAIL [18–21]. DR5 specific TRAIL lower toxicity could be attributed to negligible amount of DR5 on the surface of healthy hepatic cells in comparison to DR4 [20,22]. In this study, we investigate the potential application of two receptor-specific TRAIL variants for targeted elimination of activated HSCs.

Material and methods

Cell lines and culture

The LX-2 immortalized human HSC line was kindly provided by Prof. Scott Friedman (Mount Sinai Hospital, New York) and was cultured in Dulbecco's minimum essential medium (DMEM; Gibco, Glutamax) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 g/mL streptomycin, 50 g/mL gentamicin, and 100 nmol/L insulin. The HepG2 human hepatoma cell line (ATCC#HB-8065), and HEK-293 human embryonic kidney cell line (ATCC# CRL-1573) were cultured in DMEM (Gibco,) containing 10% fetal bovine serum. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

Primary hepatic cells

Animals Specified pathogen-free male Wistar rats were purchased from Harlan (Zeist, the Netherlands). They were housed under standard laboratory conditions and had free access to standard laboratory chow and water. Each experiment was performed following the guidelines of the local committee for care and use of laboratory animals.

Isolation and culture of rat hepatocytes and HSCs

Primary rat hepatocytes [23] and HSCs [24] were isolated and cultured as described previously. Hepatocyte viability and purity were always more than 90% as judged by Trypan blue exclusion. Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

Antibodies and TRAIL variants

The following antibodies (Ab) were used: Anti-TRAIL monoclonal Ab anti-Human CD253 (TRAIL) (eBioscience Affymetrics Co); Mouse anti- human EGFR IgG Ab; Mouse anti- human α Smooth Muscle Actin (α-SMA) IgG Ab; Goat anti- human collagen I IgG Ab; Anti-ß actin mouse IgG (Sigma). Receptor-specific TRAIL proteins DHER and 4C7 were produced and purified from prokaryotic expression systems as previously described[19,21]. For the apoptosis assay Annexin V-FITC (IQ products, IQP-120F) and Mouse IgG2b-FITC (IQ products, IQP-193F) was used. DR4-specific, DR5-specific and wild type TRAIL antibodies were used as previously described[16].

***In vitro* evaluation of receptor-specific TRAILs for inducing apoptosis in HSCs**

To activate the LX-2 cells or primary rat HSCs, cells (1000 cells /well) were seeded and incubated in 96-well, flat-bottomed uncoated plastic plates in DMEM, Glutamax (Invitrogen) media with 10% FBS for up to 7 days as previously described[25]. HSCs were exposed for 48 hours to various concentrations of WT TRAIL or receptor-specific TRAIL variants DHER or 4C7, diluted in DMEM medium. Primary hepatocytes were seeded at a concentration of (8000 cells/well) in 96-well, flat-bottomed uncoated plastic plates overnight before treatment with receptor-specific TRAILs for 48 hours. The effect of different proteins and agents on cell viability were assessed using MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium), in the presence of phenazine methosulfate (PMS)) according to the manufacturers protocol (Sigma) in triplicate. Cell only controls received DMEM 1% FBS**.**

Immunohistochemistry and Western blotting

For SDS-PAGE and Western blotting, LX-2 cells were cultured to 50% confluency. At the indicated time points TRAIL proteins were added at concentrations ranging from 10 to 100 ng/ml in DMEM. For control cells, DMEM was added. 48 hours later the supernatants were removed and the cells were washed with PBS. The cell pellets were separated on 12.5 % SDS- polyacrylamide gels as previously described[26]. For Western Blot analysis with Abs against collagen I, α-SMA and β-Actin, gels were blotted on blotting membrane as previously described [26] , followed by incubation for 2 hours at room temperature with either an Ab against human collagen I (1:1000), α-SMA (1:1000) or β-Actin (1:5000) in PBS with 1% BSA and 0.05% Tween-20. After washing, the membrane was incubated for one hour with the second Ab (horseradish peroxidase (HRP) conjugated rabbit anti-mouse IgG,; Dako, Denmark) for β-Actin and α-SMA or mouse anti-Goat IgG-HRP (Dako, Denmark) in the case of collagen I, all diluted 1:1000 in the buffer mentioned above. The membrane was then washed four times for 15 minutes in the washing solution as above. The blot was developed using an AEC staining solution system (Sigma, USA) according to the manufacturer's instructions.

For Immunohistochemistry of cultured activated LX-2 cells, first LX-2 cells were cultured on uncoated plastic in 96-well plates with 1% FCS in DMEM for 7 days, and incubated with different concentrations of TRAIL. After 24 hours the medium was removed and the cells were washed 3 times with PBS, fixed with absolute methanol (Sigma) at -20°C for 20 minutes, air-dried for 30 minutes and re-hydrated with PBS for 5 minutes. Next, the cells were incubated with either anti-collagen I

(1:75), anti-α-SMA (1:600) in PBS or 1:10,000 Hoechst 33342. Finally, the cells were washed with PBS 3 times and incubated with the second Ab (Rabbit anti-mouse IgG-HRP; Dako, Denmark) for α-SMA and (mouse-anti Goat IgG-HRP; Dako, Denmark) for Collagen I all diluted 1:100 in PBS. The integrated density of intracellular insoluble collagen or α-SMA was then calculated through analysis of five separate pictures from triplicate experiment for each treatment. All acquisition parameters were kept constant for samples and respective controls. Images were imported into Image J 1.48 software. For each wavelength back ground fluorescence has been subtracted from the selected region of interest. The integrated cell density was then calculated and normalized for the total number of cells for all pictures.

Profiling TRAIL receptor expression on HSCs

Flow cytometer analysis of TRAIL receptors was performed as follows. HSCs were detached using 0.05% Trypsin-EDTA in PBS, washed with ice-cold PBS, and diluted to a concentration of $2*10^6$ cells/mL using cold PBS. Aliquots of 100 µL ($2 * 10^5$ cells) were centrifuged at 14,000 rpm for 5 min at 4ºC, the supernatant was discarded, and the cells were separately suspended in 100 µL of rabbit anti-DR4 (10 µg/mL), rabbit anti-DR5 (10 µg/mL), rabbit anti-DcR1 (10 µg/mL) or rabbit anti-DcR2 (10 µg/mL) antibodies for TRAIL DR5 and incubated for 45 min at 4ºC. The cells were then washed twice with PBS and were incubated for an additional 45 min with 1:100 diluted anti–rabbit PE-conjugated secondary antibody under cold and dark conditions. After two final washings, cells were suspended in 300 µL PBS (containing 1%BSA). Unlabeled cells and cells labeled with secondary antibody

alone served as negative controls. The mean values of fluorescence intensity of 10,000 cells were determined by flow cytometry analysis (Calibur 1).

For analysis of TRAIL receptor distribution in activated HSC side populations (SP), LX-2 cells cultured on plastic plates for 7 days in DMEM containing 10% FBS were detached by trypsinization for 3 min. The number of viable cells was estimated by Trypan blue staining. Cells were collected by centrifugation and washed with 5 mL of phosphate-buffered saline (PBS). To detect the SP, LX-2 cells ($1 * 10^6$) were incubated with Hoechst 33342 (5 mg/mL) in DMEM/10% FBS for 90 min at 37°C with vortexing every 15 min. Cells were then stained with specific TRAIL receptor antibodies as explained above. As previously described [27] to discriminate between SP and non-SP cells, cells were incubated with the Hoechst dye in the presence or absence of verapamil (50 mM). At the end of the incubation period, 1 mg/mL propidium iodide (PI; Sigma) was added to identify dead cells before analysis by flow cytometry for Blue 450nm (blue Hoechst) and 675 (red Hoechst) laser using the BD LSR II Flow Cytometer [27]. Gated SP cells were analyzed for expression of different TRAIL receptors. All FACS data analyzed with FlowJo VX software.

RNA Isolation and Real-Time RT-PCR Analysis

Total RNA from cultured LX-2 cells was isolated using the SV total RNA isolation system (Promega Z3100) (all tests carried out in triplicate). The amount of RNA was measured by NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) and analyzed qualitatively by gel electrophoresis. Subsequently, synthesis of first-strand cDNA from total RNA was performed with Revers Transcription system (Promega A3500) in a volume of 20 µl containing 250 ng of

oligo dT (Promega, Madison, WI). The obtained cDNA was diluted with Millipore water (Millipore Corporation, Billerica, MA) to a concentration of 10 ng/µl, and 1 µl was applied for each PCR reaction. The α-SMA primer was ordered as Assays-by-Design (Applied Biosystems, Foster City, CA) (4331348/assay name ACT-R-ACT2). GAPDH was used as a housekeeping gene (Applied Biosystems). DR5- and DR4-specific receptors primers were used as previously described[25]. The PCR reaction was carried out in TaqMan PCR Master Mix (Applied Biosystems) with a final concentration of 200 nM GAPDH primers and 250 nM for primers of the other genes studied. The amplification reaction was performed in an ABI PRISM 7900HT sequence detector (Applied Biosystems) with the following cycling conditions: 2 min at 50°C, 10 min at 95°C, and 40 two-step cycles of 15 s at 95°C and 60 s at 60°C. For each sample, the real-time PCR reaction was performed in triplicate, and the averages of the obtained threshold cycle (C_t) values were processed for further calculations according to the comparative C_t method described in the ABI manual (http://www.appliedbiosystems.com). In brief, gene expression levels were normalized to the expression of the housekeeping gene GAPDH, giving the ΔC_tvalue. The average value of ΔC_t obtained from day 0 culture LX-2 was subtracted from the average of the ΔC_t value of each sample, yielding the $\Delta\Delta C_t$ value. Finally, the gene expression level was calculated as $2^{-\Delta\Delta Ct}$, giving the final value that is normalized to the housekeeping gene and relative to the control sample values of the studied α-SMA gene.

Total soluble collagen assay

The Sircol assay (Biocolor, UK) was used for measuring soluble collagen in culture supernatants. One ml of Sircol dye reagent and 150 µl of sample were mixed and incubated for 30 min at room temperature. Collagen–dye complexes were centrifuged for 10 min at 14,000rpm, washed with 1 ml of ethanol and dissolved in 1 ml of 0.5 M NaOH. The absorbance was measured at 540 nm. The assay was performed in triplicate, and the mean of data was calculated for each sample.

Caspase and Apoptosis assay

Caspase 3/7 activities were assayed using the Apo-ONE™ Homogeneous Caspase-3/7 Assay kit (Promega). Briefly, $1*10^4$ HSCs treated with 100 ng/ml of the different TRAIL variants or DMEM medium (control). Cells were transferred into a 96-well plate and 100 µl of Homogeneous Caspase-3/7 Reagent was added. The plate was covered with a plate sealer. After incubation for 2 hours, fluorescence of each well was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The measurements are reported in relative fluorescence units (RFU).

For evaluation of apoptosis, LX-2 cells were treated with TRAIL variants as described above and harvested at the indicated time points. Briefly, cells were washed with calcium buffer (0.15 M NaCl, 11.7 mM HEPES and 23 mM $CaCl_2$) buffer and re-suspended in 1/10 dilution of anti-Annexin V in 60 µL of calcium buffer. Cells were incubated for 30 minutes at 4ºC, washed in calcium buffer and re-suspended in calcium buffer supplemented with 0.5 µg/ml propidium iodate. Finally, fluorescence-activated cell sorting (FACS) was performed using the BD LSR II

Flow Cytometer. Gated cells were analyzed for apoptotic signaling of Annexin V. All FACS data were analyzed with FlowJo VX software.

Statistical analysis

All experiments were performed in triplicate and averages were used to calculate for significance between different treatments. The significance of the difference in TRAIL receptors expression was analyzed by the Student's *t* test on three separate experiments. *P* values <0.05 were considered to be significant. Comparisons between different primary cells and or cell lines treated with TRAIL variants were calculated by using a non-parametric one-ways ANOVA with Sidak's multiple comparison tests with a single pooled variance (*P < 0.05) using GraphPad Prism version 5.0 for Windows (GrahPad Software).

Results

TRAIL receptors expression increases during progressive activation of LX-2 cells in cell culture

LX-2 cells were activated by culturing the cells for 9 days on polyethylene plastic plates This model of progressive activation in culture has been established for LX-2 cells for the *in vitro* study of liver fibrosis[25,26]. LX-2 cells were harvested and screened for signs of activation via mRNA expression of α-SMA, an indicative marker of activation, during different time points. An increase of five-fold in mRNA and protein expression of α-SMA was associated with progressive activation (Fig. 1A). This model was further used to evaluate the effect of different TRAIL variants

on activated HSCs. We assessed the expression of the different TRAIL receptors using both real-time PCR and FACS analysis. Real time analysis showed that there was an increased DR4 and DR5 receptor mRNA expression in accordance with the observed activation profile for α-SMA expression in activated HSCs. The increase in mRNA expression was 100-times more for the DR4 receptor compared to the DR5 receptor (Fig. 1B). Flow cytometry analysis showed that there was a 100% and 10% shift in mean fluorescent intensity of DR4 and DR5, respectively. However, the level of DR5 expression in comparison to DR4 was 10-fold higher, which could indicate that DR5 may play a more important role in TRAIL-mediated apoptosis of HSCs (Fig. 1C) (Supplementary Fig. 1). The expression of both DcR1 and DcR2 seem to be absent on ***LX2*** cells and only detectable at low levels in activated LX2 cells.

TRAIL receptor 5 is expressed in major HSC population while TRAIL decoy receptor 2 is expressed in SP HSC population

Side populations (SP) have already been identified in bone morrow, skeletal muscles and liver cells. These cells are characterized as progenitor cells that can actively efflux the red Hoechst 33342 dye. The HSC SP has been characterized as a part of the liver SP cell type that can regenerate and transdifferentiate[28–30] . Sustainability of this cell population has been shown through long-term culture *in vitro*. Interestingly, TGF-β signaling reduces HSCs SP and renders this population susceptible to the fibrotic process where TGF-β plays a major role [17]. Due to the regenerative role of the HSC SP a better understanding of the TRAIL receptors contribution to this population could be helpful in identifying a suitable TRAIL

ligand for the treatment of fibrosis. Using the flow cytometry analysis of blue and red Hoechst dyes, we found that the HSC SP consists 1.6 % of the activated LX-2 cells (Fig. 2), The expression of TRAIL receptors on HSC SP was determined. Around 95% of HSCs expressed DR5 on their surface (Fig. 1), whereas negligible amount of SP HSCs expressed DR5 receptors on their surface. Also, around 33% and 10% of SP cells expressed DCR2 and DCR1 respectively. DCRs expression is negligible in the rest of the population (Fig. 2). These result indicated that the HSC population maintained high level of DR5 receptor expression in culture; whereas SP HSCs maintained low levels of TRAIL death receptors and higher level of TRAIL decoy receptors.

DR5-specific TRAIL decreases Extra Cellular Matrix (ECM) production by activated HSC

One of the characteristic of activated HSCs is the increased expression of fibrotic markers such as αSMA and collagen I. It has been previously been shown that TRAIL and TRAIL derivatives could reduce the expression of ECM produced by activated HSCs[26,31]. To evaluate the potential effect of receptor-specific TRAIL on collagen I expression, LX-2 cells were cultured on a plastic surface for 7 days and different amounts of receptor-specific or wt TRAIL were added to cell cultures. After 48 h, cells were analyzed for collagen production using the Sircol assay and western blotting. Both DR5 and wt TRAIL caused a substantial decrease (80%) in collagen production, even at very low dose (10 ng/ml), whereas DR4-specific TRAIL was only capable of a similar effect at a high dose (Fig. 3A). It has been reported that the expression of collagen correlates closely with the expression of collagen-specific molecular

chaperone Hsp47 in activated HSCs. Therefore, we next investigated the expression of HSP47 in LX-2 cells treated with increasing amounts of TRAIL variants. The results suggest that a decrease in collagen production was associated with down regulation of HSP47 in TRAIL-treated cells. Furthermore, DR5 receptor-specific TRAIL showed a greater capability in reducing HSP47 expression at lower concentration (Fig. 3B, C). However, the reduction of collagen was significantly higher in DR5 receptor-specific TRAIL compared to the DR4 receptor-specific TRAIL. Finally, staining of TRAIL treated LX-2 cells with anti-collagen I and anti α-SMA revealed increase in intracellular insoluble collagen is associated with decrease in α-SMA. Moreover the aggregation of insoluble collagen and reduction in the expression of α-SMA were more pronounced in cells treated with DR5-specific TRAIL, thus indicating with DR5 specific TRAIL seems to interfere more efficiently with collagen folding mechanism. (Fig. 4).

Different receptor-specific TRAILs have different potential to eliminate activated HSCs

To evaluate the potential cytotoxic effect of receptor-specific TRAIL, different amounts from each receptor-specific or wt TRAIL protein were added to activated or quiescent LX-2 HSCs and the viability of cells was determined 72 hours later by the MTS assay (Figure 5). Activated LX-2 cells showed a substantial decrease in viability by increasing doses of TRAIL. A comparison between the different TRAIL variants showed that DR5-specific TRAIL was more potent in reducing viability (60 %), whereas treatment with DR4-specific TRAIL did affect the viability of activated HSC, only in the presence of high concentrations of TRAIL (Fig. 5B). Treatment of quiescent LX-2 with different types of TRAIL, on the other hand, did

not significantly affect the viability even at the highest concentrations (1 µg/ml) (Fig. 5A). Finally, to examine the specificity of the different TRAIL variants to HSCs, the hepatic parenchymal cancer cell line HepG2 was treated with increasing concentrations of DR4, DR5 and wt TRAIL. Whereas no detectable difference in viability of HepG2 cells was observed after application of wt TRAIL, a decrease in viability was observed with the DR4 or the DR5 specific TRAIL proteins (Fig. 5C). These findings confirm the selectivity of the wt TRAIL protein towards activated HSC.

The inhibition of the viability in primary activated rat HSC was lower than in LX-2 cells and was only found at high doses of DR5-specific TRAIL (1 µg/ml), which showed a 20% reduction in viability (Fig. 5E). No significant reduction in viability was observed after treatment with the different TRAIL variants of quiescent rat HSCs (Fig. 5D) or rat hepatocytes (Fig. 5F).

We next evaluated the contribution of the different TRAIL receptor variants with respect to induce apoptosis using the caspase 3/7 activity assay. The measurement of caspase activity in HSCs treated with TRAIL showed a higher caspase activity for DR5- and WT-treated cells versus DR4-treated LX-2 cells. Data obtained were normalized to the untreated cell line (Fig. 6A).

To further investigate the apoptosis-inducing activities of the different TRAIL variants, LX-2 cells were incubated with PBS or the TRAIL variants proteins for 48 hours. Consistent with the cell viability results, only wt and DR5-specific TRAIL induced robust apoptosis of LX- 2 cells, whereas DR4- specific TRAIL was less effective (Fig. 6B) as measured by Annexin V FACS analysis.

Discussion

Persistent liver injury lead to enhanced ECM production and accumulation, a condition referred to as liver fibrosis. HSCs have a central role in this process, , it has been shown that activated HSCs significantly amplify the hepatic response to liver injury, hence depleting fibrotic livers from activated HSCs may ameliorate the fibrotic condition [32]. TRAIL-induced apoptosis was successfully used to eliminate activated HSC [25]. In order to increase the specificity and to be able to lower the effective dose of TRAIL we evaluated the different properties of receptor-specific TRAILs as a selective treatment for the elimination of activated HSCs to improve the remedy from liver fibrosis. To uncover the dynamics of TRAIL receptor expression during time-dependent HSC activation, the expression pattern of different TRAIL receptors on the surface of quiescent and activated HSCs was evaluated. Our findings cast light on the differential contribution of TRAIL receptors in HSCs. An increase in the level of TRAIL DR5 and DR4 receptors was observed both in mRNA and protein expression upon activation of HSCs (Fig. 1). Despite the increase in TRAIl-DR4 receptor expression during activation, the net expression remained significantly lower than that of the TRAIl-DR5 receptor. An increase in the expression of TRAIL receptors was concomitant with an increase in expression of TRAIL decoy receptors, especially DcR2. As part of our study we demonstrated that the DR5 receptor was the single most dominant receptor on HSCs in comparison with the HSC side population, whereas decoy receptor DcR2, was predominantly expressed on the surface of HSC side population. This finding is particularly important since It has been proven that HSCs may directly undergo

mesenchymal-to-epithelial transition to transdifferentiate into liver progenitors [25]or indirectly augment regeneration of hepatic cells by its produced cytokines [26][27]. The exact mechanisms by which HSCs may help mediate liver regeneration and the relative importance of different subtypes of HSCs remain to be determined. However, crucial regenerative role of the SP in multiple organs imply the possibility for this population to have similar role in liver regeneration. Thus, next to the higher expression of TRAIL receptors on activated HSCs the selectivity of TRAIL variants could be partially explained by the higher expression of the decoy receptors in SP HSCs than in rest of population. Our findings therefore, show the protective role of TRAIL decoy receptor 2 in HSCs SP.

To further evaluate the application of different receptor-specific TRAIL agonists in eliminating activated HSCs we explored the functional capacity of receptor-specific TRAIL variants [15,16][36] in inducing apoptosis and reducing ECM production in HSCs. ECM-producing cells, such as HSC, are highly responsive to a series of factors such as EGF , PDGF and TGF-β that are released from damaged liver parenchyma during the fibrotic process. Affected HSCs start producing excessive amount of ECM. The production of ECM, especially Collagen I, could in return accelerate HSC proliferation and fibrosis [37,38]. Addressing this phenomenon, we evaluated the capability of different TRAIL variants in blocking ECM production by HSCs. All TRAIL variants suppressed the production of soluble collagen by activated HSCs with DR5 receptor-specific and wt TRAIL being the most potent molecules. This observation was accompanied by down regulation of the pro-collagen specific molecular chaperone Hsp47 and aggregation of insoluble collagen

in TRAIL-treated cells. These findings cast light on the supporting mechanism of receptor-specific TRAIL mediated collagen regulation through interfering with the natural folding and secretion process of pre-collagen in activated HSCs. A direct relation has already been established between fibrogenesis and the number of α-SMA positive cells [39]. Since the increase in accumulation of insoluble collagens in our study was associated with a reduced number of α-SMA positive cells, it may be speculated that the decreased expression of ECM proteins is caused by the reduction in HSC activation and proliferation. The exact mechanism for this phenomenon yet to be defined, but it has been shown that TRAIL could inhibit cellular growth in the absence of cell death induction due to cyclin dependent cell cycle arrest in S-G2/M phase[40–42]. Hence, inhibition of HSCs activation and proliferation by TRAIL could directly reduce secretion of ECM including collagen I. An alternative mechanism for reduced activation of TRAIL affected HSCs could be attributed to role of pericellular collagen on HSCs proliferation. There is accumulating evidence that HSC biology is tightly regulated by their peripheral ECM environment. It has been shown collagen I, the major component of fibrosis, enhances the proliferation of HSC *in vitro* via different mechanisms i.e. interacting with type 2 discoidin domain receptor [43,44]. Thus, TRAIL property for *in vitro* eliminating ECM accumulation through interfering with pro-collagen folding and secretion could eliminate HSCs proliferation and promote reversion of the fibrotic phenotype of activated HSCs[45].

Our findings revealed a substantial decrease in LX-2 HSC viability achieved through exposure to DR5-specific and wt TRAIL, whereas DR4-specific TRAIL showed

only a marginal effect on LX-2 cell viability. This finding is in concordance with findings regarding the functional role of different TRAIL variants in inducing apoptosis as measured by increase in caspase 3/7 and Annexin V [25]. Selectivity of TRAIL variants is an important issue since apoptosis in itself could have a dual function in ameliorating or deteriorating fibrosis progression. Here, we showed that the effects of receptor specific TRAIL variants are limited to the HSCs rather than the hepatocytes. The observed sensitivity of HepG2 cells towards receptor-specific TRAIL could be partially explained through its over expression of pro-apoptotic TRAIL receptors and DcR2 in hepatocellular carcinoma cell line [46]. Since fibrosis is considered rather a long term and persistent problem, a durable and efficient approach need to be pursued. Our current strategy to dissect the functional properties of different TRAIL variants is particularly beneficial since TRAIL shows a very short half-life (6 hours) *in vivo* and thus needs high dosages and frequencies of administration for clinical benefit. We speculate that an advantage of treatment with receptor-specific TRAIL variants is the specific targeting of activated HSCs and lowering required dosage for having the same effect. Moreover, given that TRAIL variants carry both separate functions for simultaneously inducing apoptosis through TRAIL receptors and reducing ECM production via blocking relevant signaling pathways in HSCs, it appears to be an ideal combination for eliminating fibrotic process. Hence, a loss of viability in TRAIL treated activated HSCs could be followed by a lower proliferation and reversion to quiescent phenotype of remaining activated HSC. This is particularly important for sustaining the effect of TRAIL. Furthermore, TRAIL variants may be applied as genetic therapy [47] resulting in

highly effective long-term *in vivo* elimination of activated HSCs and reduction of ECM production. In this regard, our study is the first to show a significant anti-fibrotic effect of different receptor-specific TRAIL variants, which warrants further *in vivo* studies.

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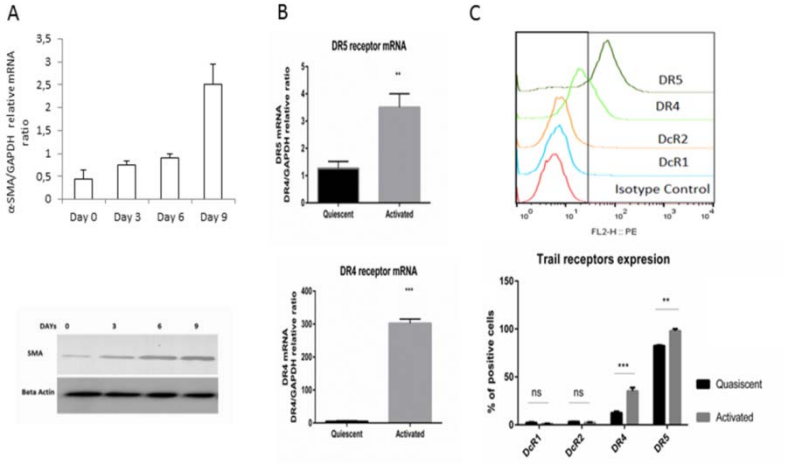


Figure 1: LX2 cells activation and TRAIL receptor expression profile. Alpha SMA mRNA and protein expression increase as a sign of activation due to progressive culture of LX2 cells *in vitro* during 9 days(A); (B) mRNA expression profile of TRAIL receptors 1 and 2 in activated LX2 cells by real time PCR; (C) Flow cytometry analysis of TRAIL receptors in LX2 cells. An increase in the expression of surface receptors is associated with the activation of HSCs during culture induced activation.ns; not significant, *P* values <0.05 were considered to be significant *, *P* values <0.005 **; <0.0005 highly significant ***.

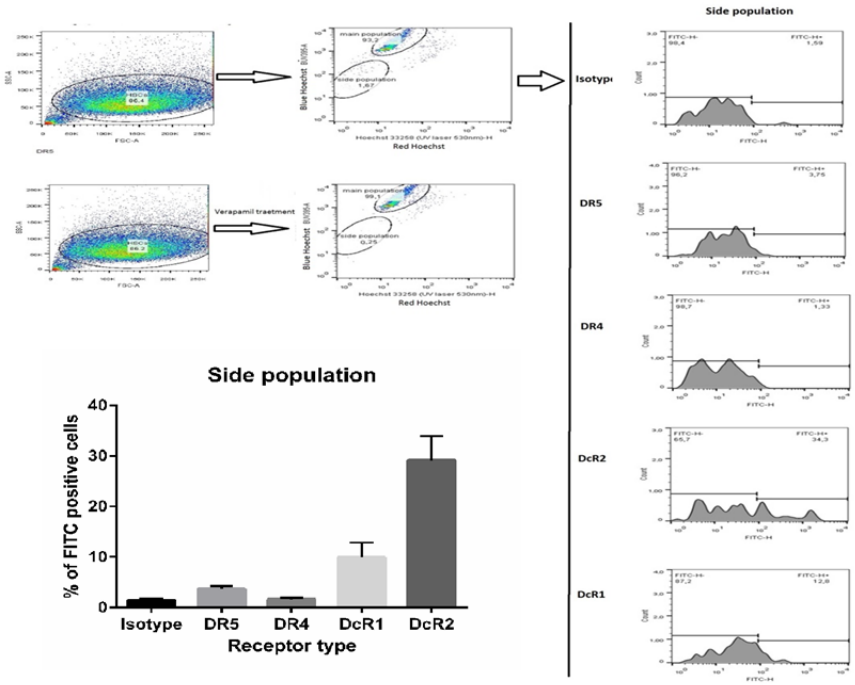


Figure 2: FACS analysis profiling of TRAIL receptors expression on surface of side population LX2 cells. LX2 cells were stained with Hoechst 33342 and different TRAIL receptor specific antibodies. Sorted side population cells were separately gated against each specific TRAIL receptors.

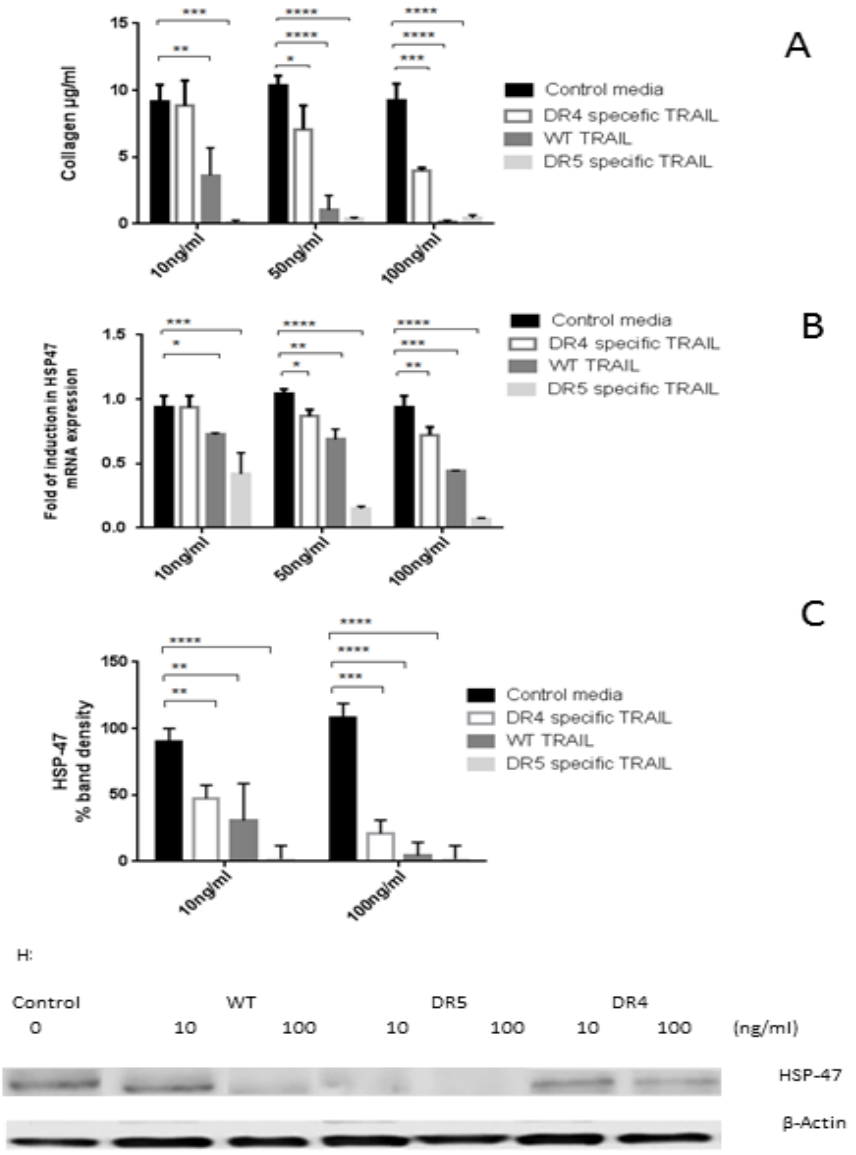


Figure 3: Interfering in LX2 cells collagen production by receptor specific TRAIL variants. Representative Sircol assay for collagen (A); HSP47 mRNA expression (B) and (C) protein production in LX2 cells treated with three different concentration of TRAIL proteins (*P* values <0.05 were considered to be significant *, *P* values <0.005 **; <0.0005 highly significant***).

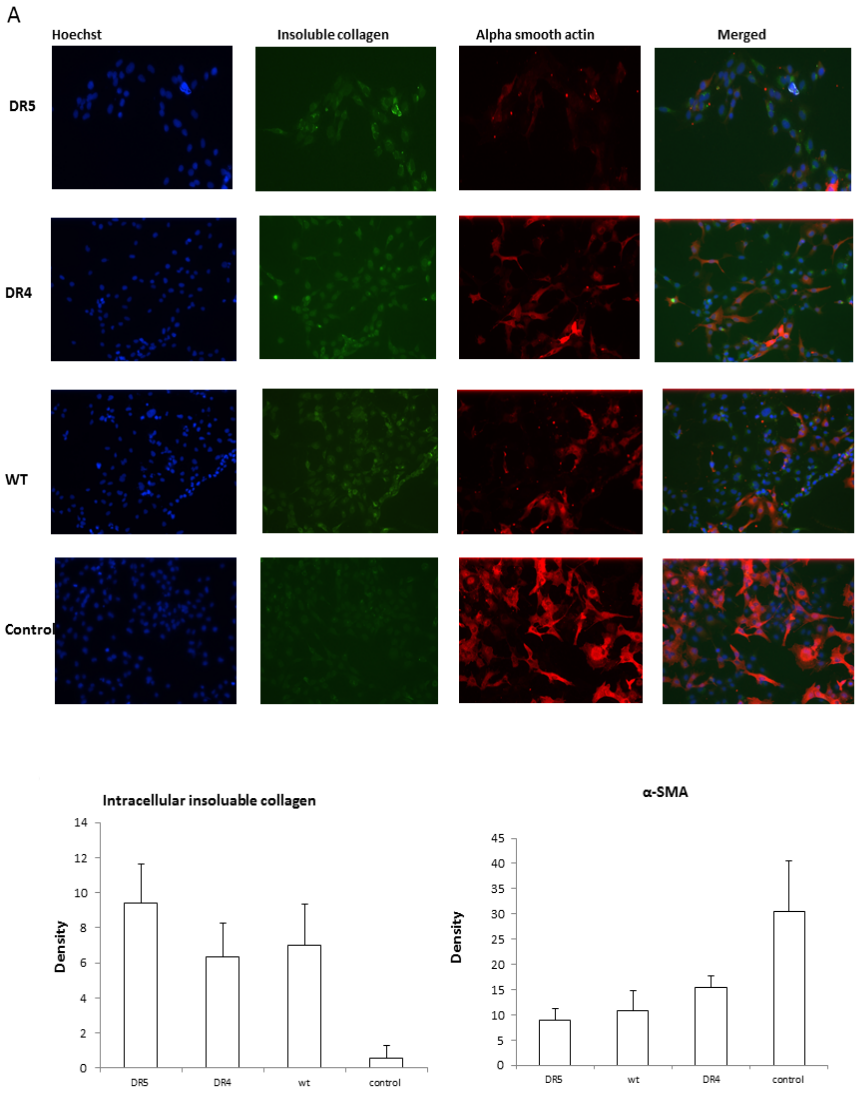


Figure 4; Immunohistochemistry staining of LX2 cells treated with TRAIL proteins showed a decrease in number of αSMA positive cells and an increase in intracellular insoluble collagen deposition. Activated LX2 cells treated with 50 ng/ml of different TRAIL proteins or DMEM (control) for 48 hours and stained for collagen (Green) or α-SMA (Red) and Hoechst nuclear staining (Blue)(A); integrated density of intracellular insoluble collagen and α-SMA in LX2 cells affected by different TRAIL or DMEM (control)(B).

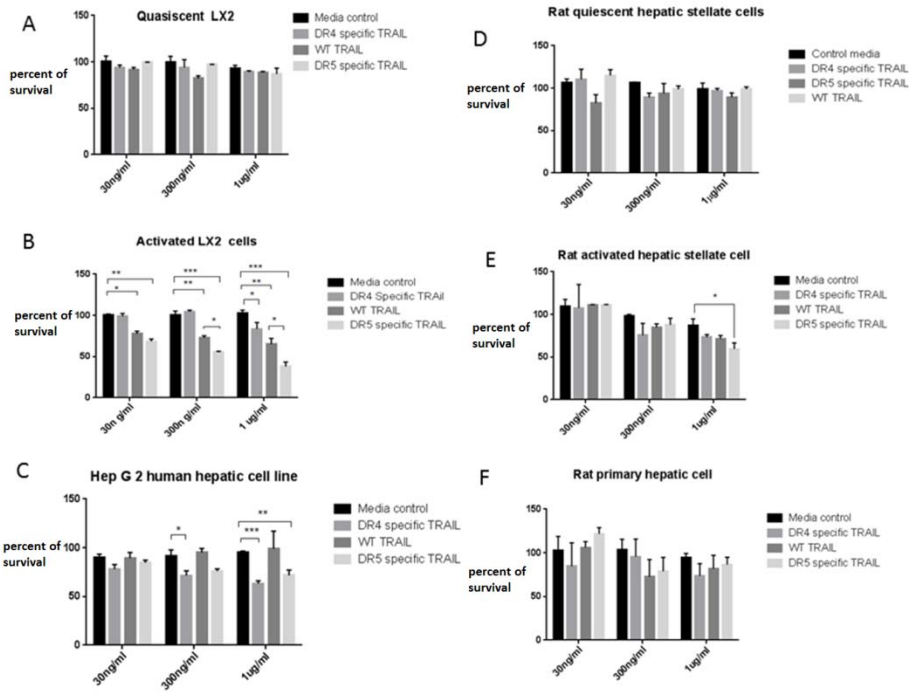


Figure 5; TRAIL efficiency in eliminating activated HSCs viability differs depends on TRAIL variants. MTS viability assay for quiescent LX2 cells (A); activated LX2 cells (B); Hep-G2 cell line (C); quiescent rat primary HSCs (D); activated rat primary HSCs (E); rat hepatocytes (F).

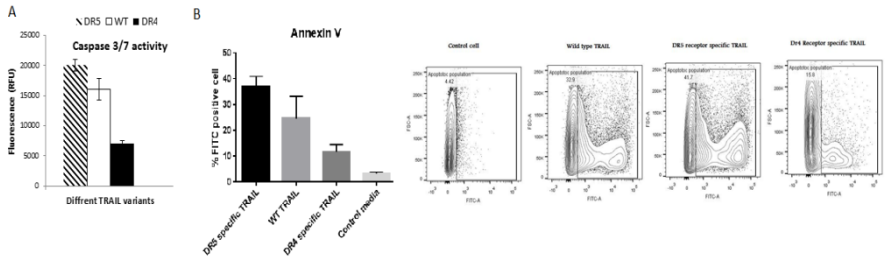
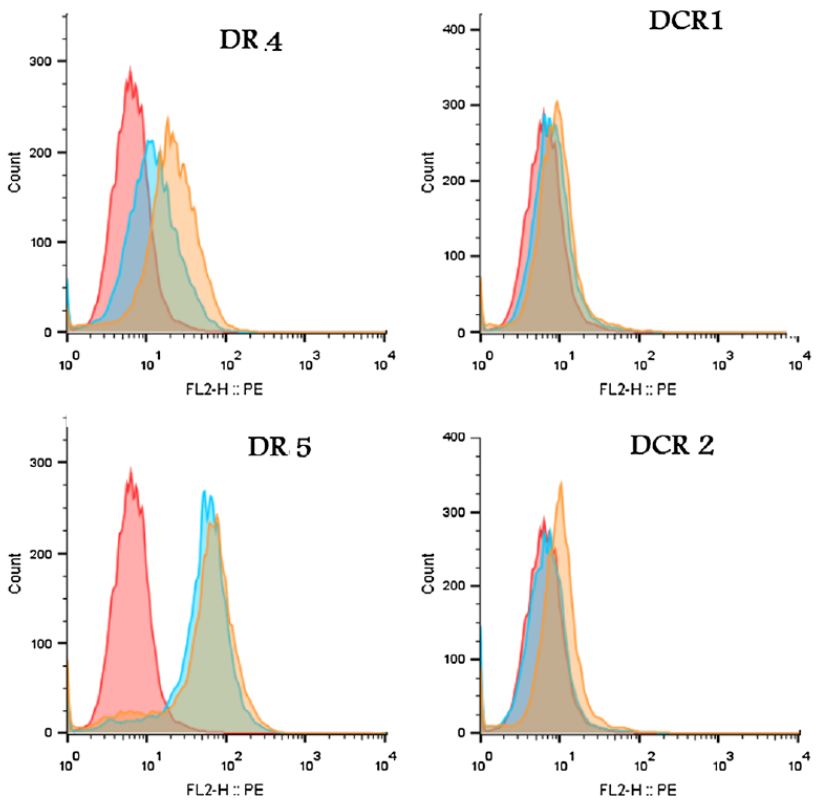


Figure 6; Comparison of caspase 3/7 enzyme activity and Annexin V in activated HSCs treated with different TRAIL variants. An increase in the amount of caspase-3/7 activity in LX2 cells (A); treated with TRAIL variants is concurrent with increase in apoptosis marker Annexin V in FACS analysis (B).



Supplementary Fig. 1 TRAIL receptors expression varies after activation in HSCs. S1 A: TRAIL receptors DR5, DR4 DcR2 and DcR1 expression in **quiescent** (Blue) vs Activated (Brown) HSCs. Isotype control (Pink).

Chapter 5

Targeting human carcinoma cells by receptor-specific TRAIL and Histone acetyl transferase / Deacetylase inhibitor regimens drives enhanced killing efficiency

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Abstract

Background Epigenetic events, including histone acetylation modifications, have been demonstrated to have critical roles in the therapeutic outcome of Tumor Necrosis Factor Related Apoptosis-Inducing Ligand (TRAIL) application in eliminating malignant tumors. Histone acetyl transferase and deacetylase (HDAC and HAT) enzymes are known to play the central role in the process of histone acetylation modification. In the present study, we investigated the potential application of a combining novel HDAC or HAT inhibitors with TRAIL variants in killing human carcinoma cells.

Methods A panel of different carcinoma cell lines (colon, lung and hepatocellular carcinoma) was treated with different TRAIL variants in presence of different HDAC or HAT inhibitors to evaluate its effect on the viability of these cell lines.

Result *In vitro* treatment of human carcinoma cells with different receptor specific and wt TRAIL variants induced a reduction in viability with DR4 specific TRAIL being the most efficient. However, a significant impairment in cell viability was observed during administration of TRAIL in presence of HDAC inhibitor SAHA or HAT inhibitor C646.

Conclusion Here, we present evidence for the successful application of the receptor specific TRAIL variants in the elimination of human carcinoma cells via simultaneous inhibition HAT or HDAC enzymes. The combined HAT or HDAC inhibitor and TRAIL variant regimen may thus represent a new therapeutic compound against different human carcinoma.

Introduction

Since TRAIL protein has been introduced to the field of tumor therapy it has raised many hopes for specific elimination of tumor cells. The application of TRAIL to induce apoptosis in tumors is mainly grounded on up-regulation of TRAIL receptors in tumor cells versus normal cells. TRAIL has two dedicated receptors (DR5 and DR4) that signal apoptosis via binding to TRAIL agonists. However, up-regulation of decoy receptors (DCR1 and DCR2) in tumor cells could lead to abortive signaling and impaired apoptosis. This later is considered the primary source of TRAIL resistance and tumor evasion. To address this problem receptor specific agonist TRAIL has already been introduced both in the form of type specific mutant TRAIL agonists or monoclonal antibody against specific TRAIL receptors DR4 and DR5 [1][2][3]. Dynamic of wild type (wt) occurring TRAIL and mutant TRAIL interaction with their receptors have already been well characterized[4]. Receptor specific agonists could reduce the decoy receptor-mediated antagonism, hence a cumulative effect by using receptor specific TRAIL is lowering required administrated dose with possibly fewer side effects that attached to TRAIL adverse effect [4][5]. Epigenetic alterations could also lead to defective apoptotic signaling and develop TRAIL resistance in tumor cells. Histone acetylation is the result of the balance between the activity of histone deacetylases (HDAC) and histone acetyl transferases (HAT). Extent of histone acetylation determines the extent of chromatin relaxation hence plays a major role in the regulation of gene expression. Deregulation of HAT or HDAC in tumor cells is associated with the failure to undergo apoptosis in cancer cells. Through chromatin condensation HDAC could

repress the expression of tumor suppressor and pro-apoptotic genes and confer resistance to apoptosis [6][7][8] , whereas HATs affect the chromatin remodeling and can promote expression of cancer related proto-oncogenes in cancer[9][10][11]. Extensive studies have demonstrated synergistic effects of HDAC inhibitors and TRAIL on apoptosis [6][7][8]. Unlike HDAC inhibitors, the interactive role of HAT inhibitors and TRAIL on apoptosis have been less characterized. In this study we investigate the potential application of TRAIL variants in combination with HDAC and HAT inhibitors to eliminate cancer cells.

Methods and materials

Cells and cultures

SW948 [SW-948] (ATCC CCL-237) Dukes' type C, grade III, colorectal adenocarcinoma, was cultured in Leibovitz's L-15 Medium (Sigma-Aldrich) supplemented with 10% Fetal calf serum. NCI-H460 [H460] (ATCC HTB-177) Human lung carcinoma cell line cultured in RPMI-1640 (Sigma-Aldrich) Medium supplemented with 10 % fetal calf serum. HepG2 [HEPG2] (ATCC HB-8065) hepatocellular carcinoma cell line cultured in DMEM high glutamine (Glutamax) (Sigma-Aldrich) supplemented with 10 % fetal calf serum. Huh-7 cell line cultured in DMEM high glutamine (Glutamax) (Sigma-Aldrich) supplemented with 10 % fetal calf serum. All the cells were grown in 37°C in presence of 5 % Co2.

Chemicals

Receptor specific or wild type TRAIL were produced and purified from prokaryotic expression as previously described [1][2][3]. Crystal violet solution (Sigma-

Aldrich), HAT inhibitors C646 Ca t# S7152 and MG149 cat # S7476 (Selleckchem). HDAC inhibitors; SAHA **N**-hydroxy-N′-phenyl-octanediamide, Suberoylanilide hydroxamic acid, Vorinostat, cat #SML0061 and MS-275 A HDAC1 and HDAC3 inhibitor Synonym: **3-**pyridinylmethyl[[4-[[(2-aminophenyl) amino] carbonyl] phenyl] methyl] carbamate, MS-275 (Entinostat, SNDX-275), N-(2-Aminophenyl) -4- [N -(pyridine-3 etylmethoxycarbonyl) naminomethyl] benzamide cat # EPS002 (Sigma-Aldrich).

Crystal violet viability assay

Then, 100 µl of a cell suspension 10 4 cells/ml for mentioned cells were gently introduced into each well from 96 well plates. After being kept undisturbed at room temperature for 20 min to allow the cells to sediment, the plates were transferred to a CO2 incubator and cultured for 2 days. The cells then were treated with 100 µl of medium containing different combination of wt and receptor specific TRAIL in presence or absence of HAT or HDAC inhibitors for hours. For cell fixation each well received 50 ul glutaraldehyde (25 %) and was left for at least 20 min. After being washed with water, the plates were stained with 0.4 % crystal violet solution in methanol for 30 min. Absorbance at 590 nm was measured by an automatic microtiter plate reader. Average absorbance of the control wells, which received no chemical, was regarded as 100 %, and the percentage of cell growth in each well was calculated.

Results

HAT and HAC inhibitors markedly potentiate TRAIL lethality in colon carcinoma cell line

To evaluate the effect of receptor specific TRAIL, different amounts from receptor specific or wt TRAILs were added to SW948 cells and the viability of cells was determined 24 hours later by the CVS assay. SW948 cells showed slight decrease in viability occurred in the presence of different TRAIL variants at sub-lethal concentration. Also comparison between different concentrations of SAHA and Entinostat showed SAHA displays a greater potential in reducing viability of SW948 cells (Fig 1). Co-treatment of SW948 cells with different type of TRAIL and HADAC inhibitors significantly increased SW948 sensitivity to apoptosis (70 % and 90 % in order for Entinostat and HDAC) at the sub-lethal concentration (10ng/ml) of TRAIL variants (Fig. 1). We next evaluated the contribution of HAT inhibitors with respect to their effect on increasing SW948 cells sensitivity towards TRAIL variants. Measurement of viability in SW948 cells treated with TRAIL variants (10ng/ml) in presence of MIG 149 and C646 showed a decrease of 40 % and 80 % respectively in compare with cells treated only with TRAIL variants (Fig. 2).

Potentiation of apoptosis in H460 lung carcinoma exposed to TRAIL and HAT/HDAC inhibitors

To further investigate the apoptosis-inducing activities of different TRAIL variants, H460 lung carcinoma cells were incubated with PBS or the aforementioned TRAIL variants proteins. Consistent with cell viability results, only DR4 specific TRAIL induced robust apoptosis of H460 cells (90 %), whereas wt and DR5 specific TRAIL

were less effective in reducing viability at lower concentration of 10 ng/µl (Fig. 3). In marked contrast to results obtained following exclusive treatment of cells with HDAC inhibitor drugs, simultaneous exposure of cells to a sub-toxic concentration of TRAIL (10 ng/ml) in conjunction with 1uM Entinostat or SAHA resulted in a very dramatic decrease in cell survival (Fig. 3). To determine whether activators of the extrinsic pathway could similarly enhance HAT inhibitor-associated lethality, H460 cells were simultaneously exposed to either C646 or MG149 in combination with different TRAIL variants. As shown in Fig. 4, while DR5 receptor specific TRAIL alone was minimally toxic, co-administration of HAT inhibitors and in particular C646 resulted in marked increase in the extent of cell survival loss. The maximum cytotoxic effect was achieved by combining 10 uM C646 and TRAIL (10 ng/ml) (Fig .4).

Co-administration of TRAIL with HDAC inhibitors potently induces apoptosis in human hepatocellular carcinoma cell lines

To determine whether the previous findings were restricted to colon or lung carcinoma cells, parallel studies were performed using human hepatocellular carcinoma cell line; Huh-7. Cells were exposed for 24 h to 1 or 5 -mM Entinostat or SAHA in presence of 10ng or 100-ng/ml TRAIL variants after which cell death was assayed. Responses to HDAC inhibitors given individually varied between the administered drugs with Huh-7 cells showed greater sensitivity to SAHA in compare to Entinostat. Simultaneous exposure of cells to TRAIL in conjunction with HDAC inhibitors resulted in a clear increase in apoptosis, comparable with results obtained in cells only treated with SAHA. This effect however was most pronounced for DR4

TRAIL ligand treated cells (Fig. 5). In the same way administration of HAT inhibitors showed that Huh-7 cells showed greater sensitivity to C646 in compare with MG149. Co-administration of C646 and DR4 TRAIL promises to be the most effective combination in inducing apoptosis in Huh-7 cells (Fig. 6).

Conclusion

TRAIL agonists are vastly used to kill malignant cells that express TRAIL receptors. The extrinsic pathway of apoptosis is initiated by the binding of death receptors, including Fas (CD59), TNF receptor-1 (TNFR-1) and TRAIL receptors (DR-4 and -5) to their correspondent ligands and the subsequent activation of caspase-8 and caspase-10[12]. However, usefulness of TRAIL in inducing apoptosis could be hampered by the resistance of some cancer cells to TRAIL. Of all known mechanism of TRAIL resistance epigenetic events, including histone acetylation modifications, have been demonstrated to have critical roles in developing resistance toward TRAIL through different mechanisms.

The present findings indicate that simultaneous administration of TRAIL with HDAC inhibitors induces apoptosis through sensitization of malignant cells to TRAIL effect. Treatment a panel of carcinoma cell lines (Lung, colon and hepatocellular) with TRAIL variants in presence of SAHA drastically enhance cells sensitivity to apoptosis. In contrast, application of Entinostat did not significantly increase TRAIL sensitivity. Importantly the results emphasize SAHA sensitization of TRAIL-resistant cells achieved with the lowest concentration of SAHA and

TRAIL that otherwise were not effective alone. However, the main question remains as to how combined treatment with SAHA and TRAIL resulted in such a significant impairment in cell survival. In fact the cooperative effects of SAHA and TRAIL could be interpreted in several levels; HDAC inhibitors can upregulate the expression of death receptors and their ligands in transformed cells, yet not in normal cells [13][14]. TRAIL receptors DR-4 and DR-5 were induced both *in vivo* [14][15] and *in vitro* [16] by different types of HDAC inhibitors[17][18][19][20][21]. On the basis of our results, receptor specificity of TRAIL variants did not significantly contribute to their killing effect in presence of SAHA. This later could indicate HDAC inhibitors mechanism of action in sensitizing cells is independent of a specific TRAIL receptor up-regulation. HDAC inhibitors could also amplify TRAIL killing effect in cancer cells through other mechanisms including; up-regulation of caspase-8, down-regulation of anti-apoptotic molecules like cFLIP and Bcl-2 proteins (e.g., Bcl-2, Bcl-XL, and Mcl-1), increase in pro-apoptotic Bcl-2 proteins (e.g., Bid, Bim, and Bax), and re-distribution of TRAIL receptors in lipid rafts on the surface of targeted cells [22][23][24] . However, the exact mechanism of this phenomenon needs to be further investigated. Unlike HDAC inhibitors, less extensive studies have been carried out to unravel the role of HAT inhibitors in TRAIL-induced apoptosis. Selective inhibition of HAT inhibits the DNA damage response in malignant cell lines [11]. Here we evaluate cytotoxicity induced by pretreatment of multiple myeloma cells with C646 or MG149 followed by TRAIL using CVS cell proliferation assay. Whereas MG149, low concentration of C646 (HAT inhibitor)

augments TRAIL-induced Cytotoxicity in multiple carcinoma cell lines. Indirect evidence suggests some level of regulation on TRAIL receptor expression due to effect of HAT inhibitors. For instance P53 a key factor that increases as a result of inhibiting P300/BCP (histone acetyltransferase) could upregulate DR5 receptor in conjunction with NF-κB[15][25][26][27]. Selective inhibition of P300 HAT via C646 HAT inhibitor could also decrease expression of a series of genes including Ubiquitin-like with PHD and ring finger domains (UHRF1) (responsible for G1/S transition and p53-dependent DNA damage checkpoint), DEP domain containing (DEPDC1) (responsible for inhibition of apoptosis) up to 14 times; while increasing the expression of P53 gene that is responsible for response to diverse cellular stresses and apoptosis senescence 1.2 times [28][29][30]. In conclusion, present evidence indicates that TRAIL in conjunction with HDAC or HAT inhibitors constitutes a potent apoptotic stimulus in human carcinoma cells. Previous studies have demonstrated that co-administration of HDAC inhibitor with TRAIL enhance the antitumor activity of TRAIL [6][7][8]. The present results suggest that similar interactions occur in human carcinoma cells exposed to TRAIL in combination with HAT inhibitors. Given that there is a growing interest in combined regimen for more efficient application of TRAIL in anti-tumor therapy, the current finding is the first to show a significant anti-tumor concept of combining TRAIL variants with HAT inhibitors, which warrants further detailed studies *in vivo*.

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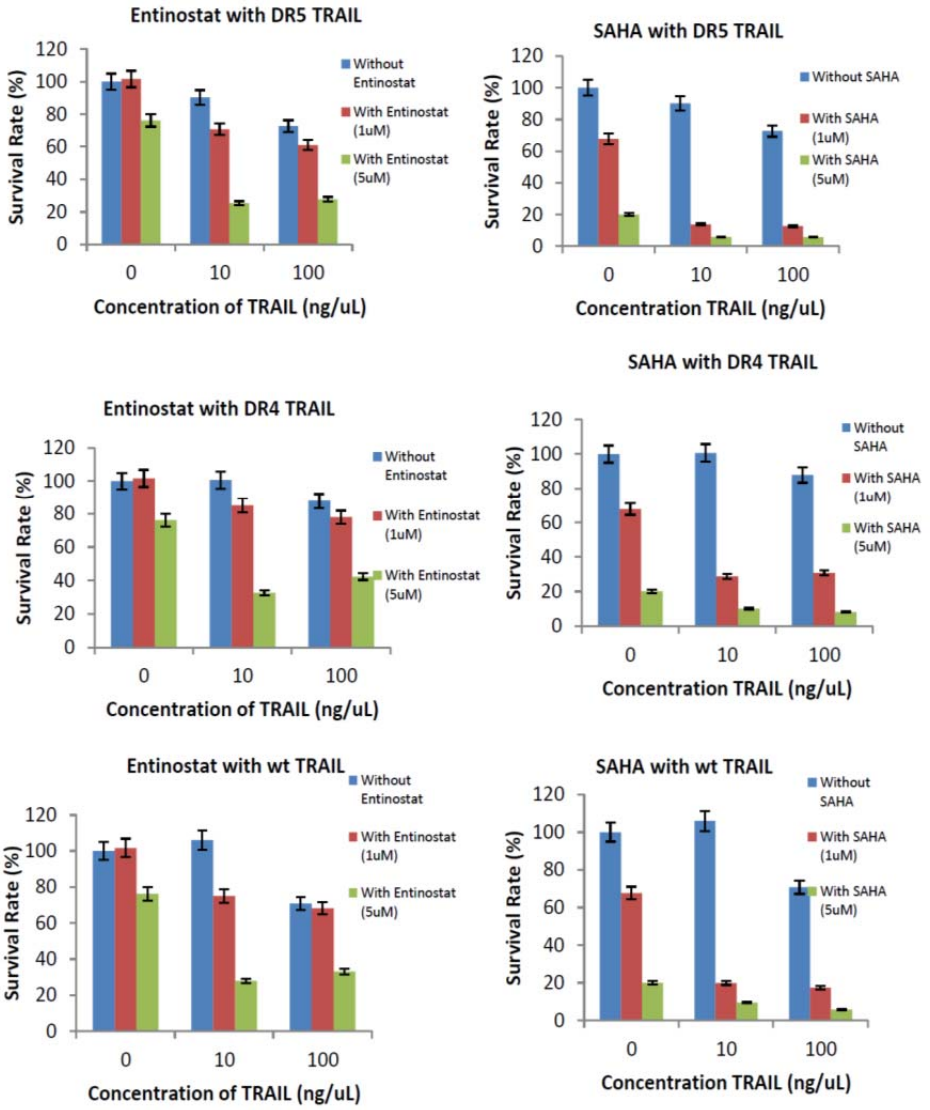

Figure 1: Simultaneous application of histone deacetylase inhibitor and TRAIL enhances the efficiency of TRAIL variants in SW948 colon carcinoma cell line.

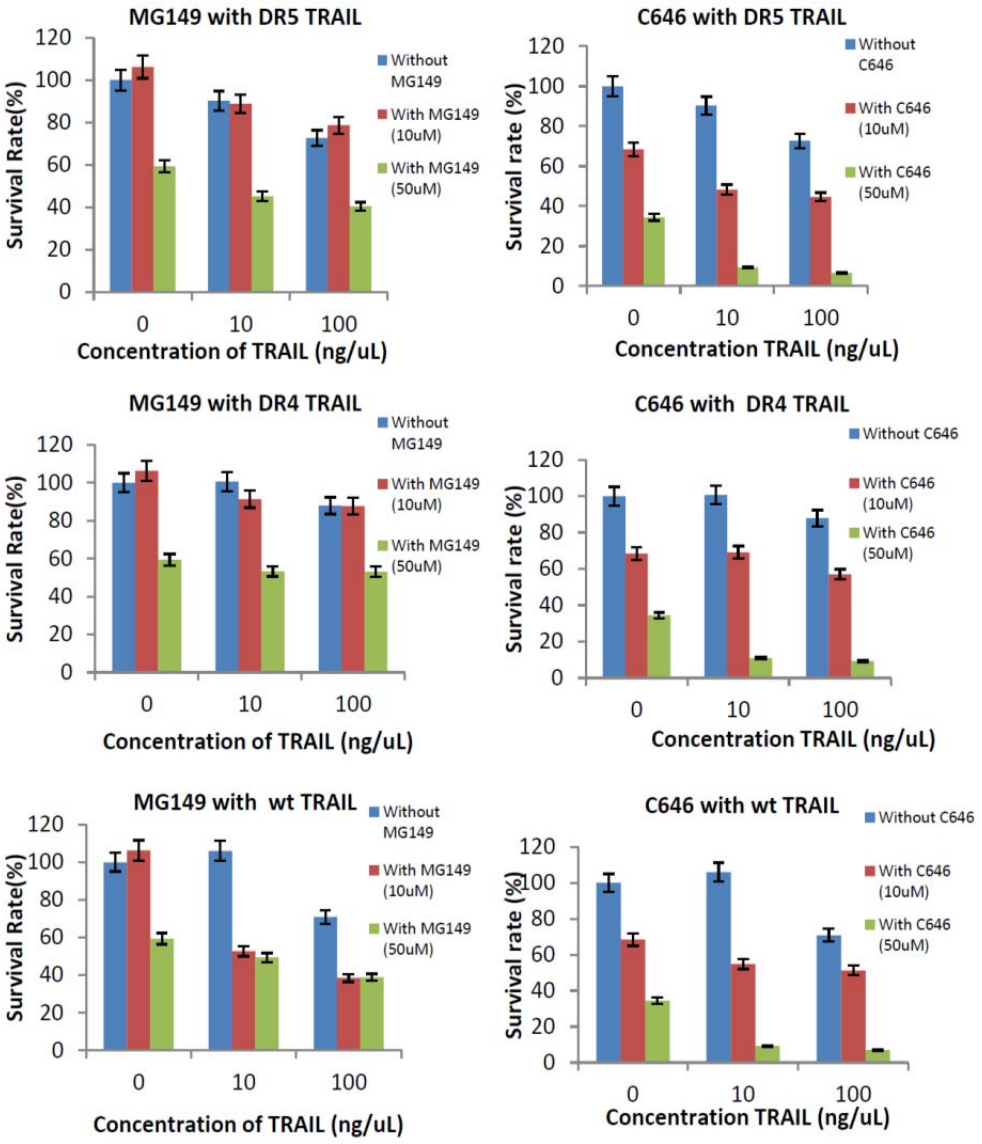

Figure 2: Simultaneous application of HAT inhibitor and TRAIL enhance the efficiency of TRAIL variants in SW948 colon carcinoma cell line.

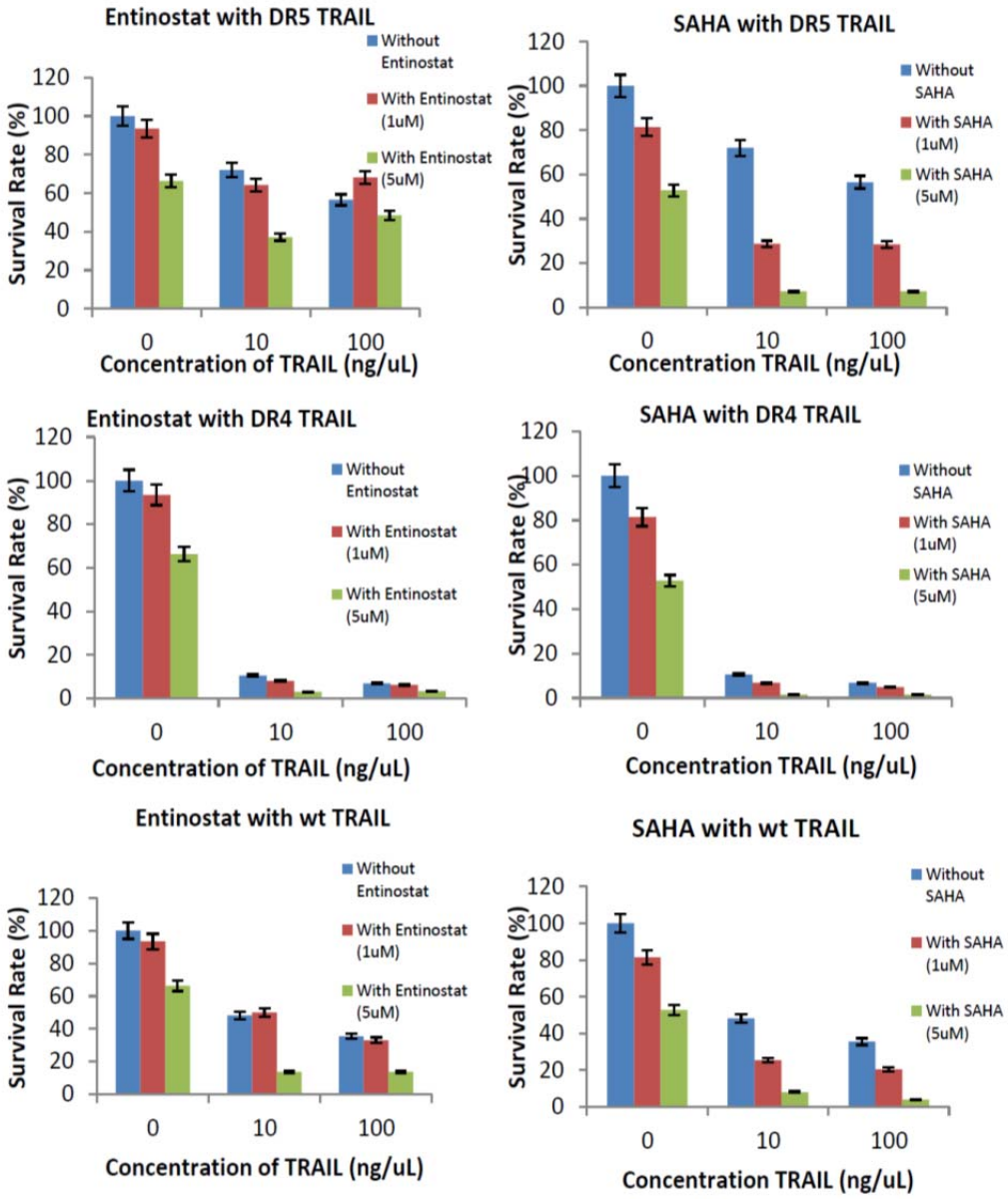

Figure 3: Simultaneous application of histone deacetylase inhibitor and TRAIL enhance the efficiency of TRAIL variants in H460 (lung carcinoma) cell line.

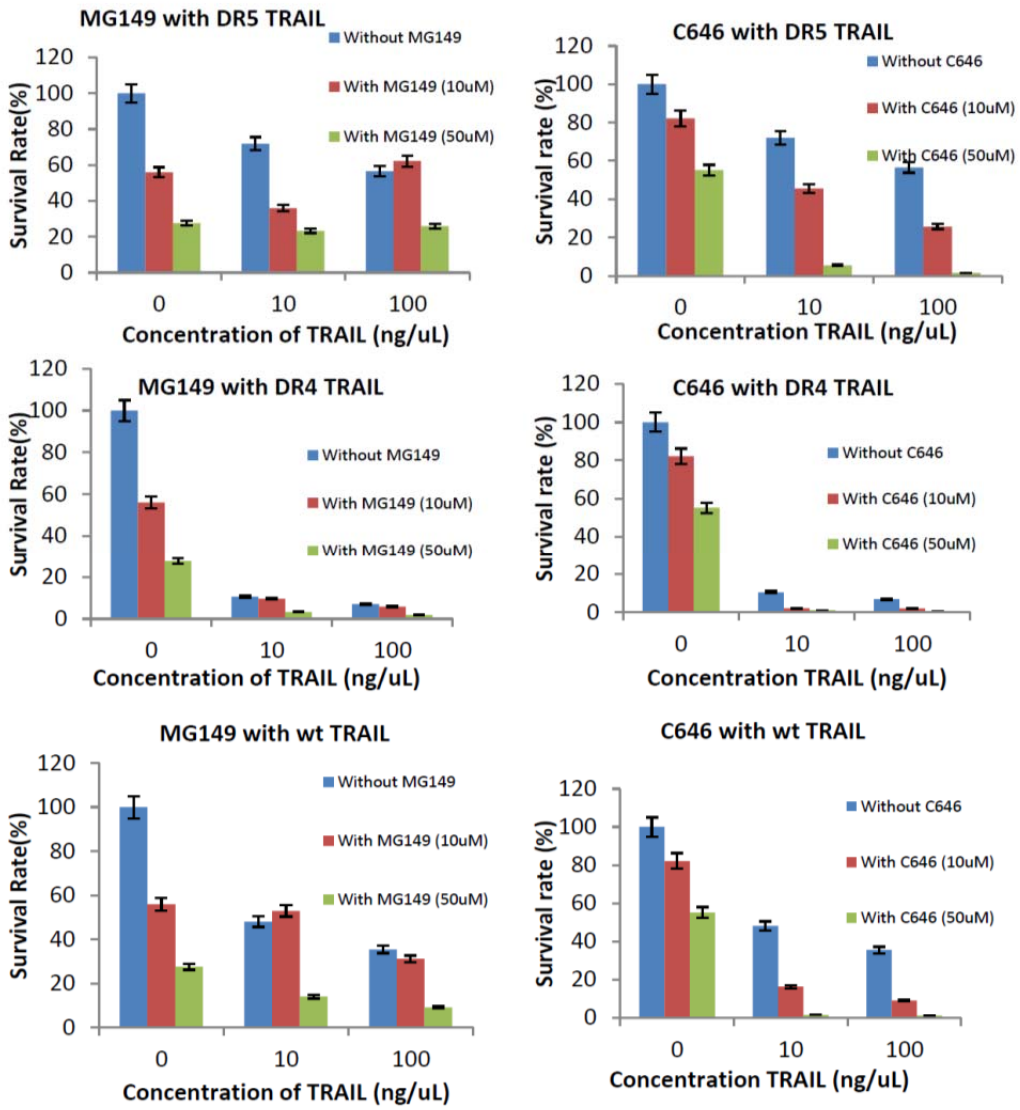

Figure 4: Simultaneous application of histone acetylase inhibitor and TRAIL enhance the efficiency of TRAIL variants in H460 cell line.

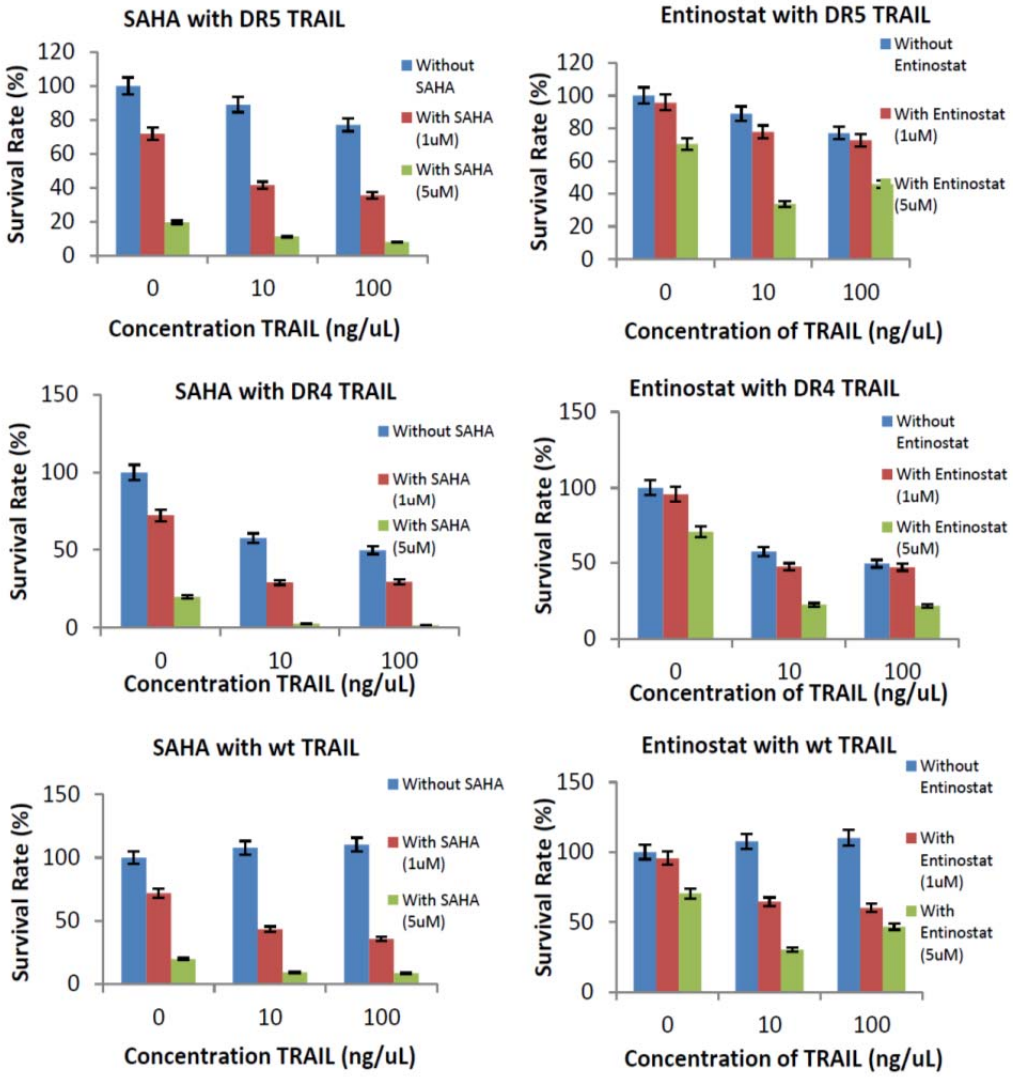

Figure 5: Simultaneous application of histone deacetylase inhibitor and TRAIL enhance the efficiency of TRAIL variants in Huh-7 (hepatocellular carcinoma) cell line.

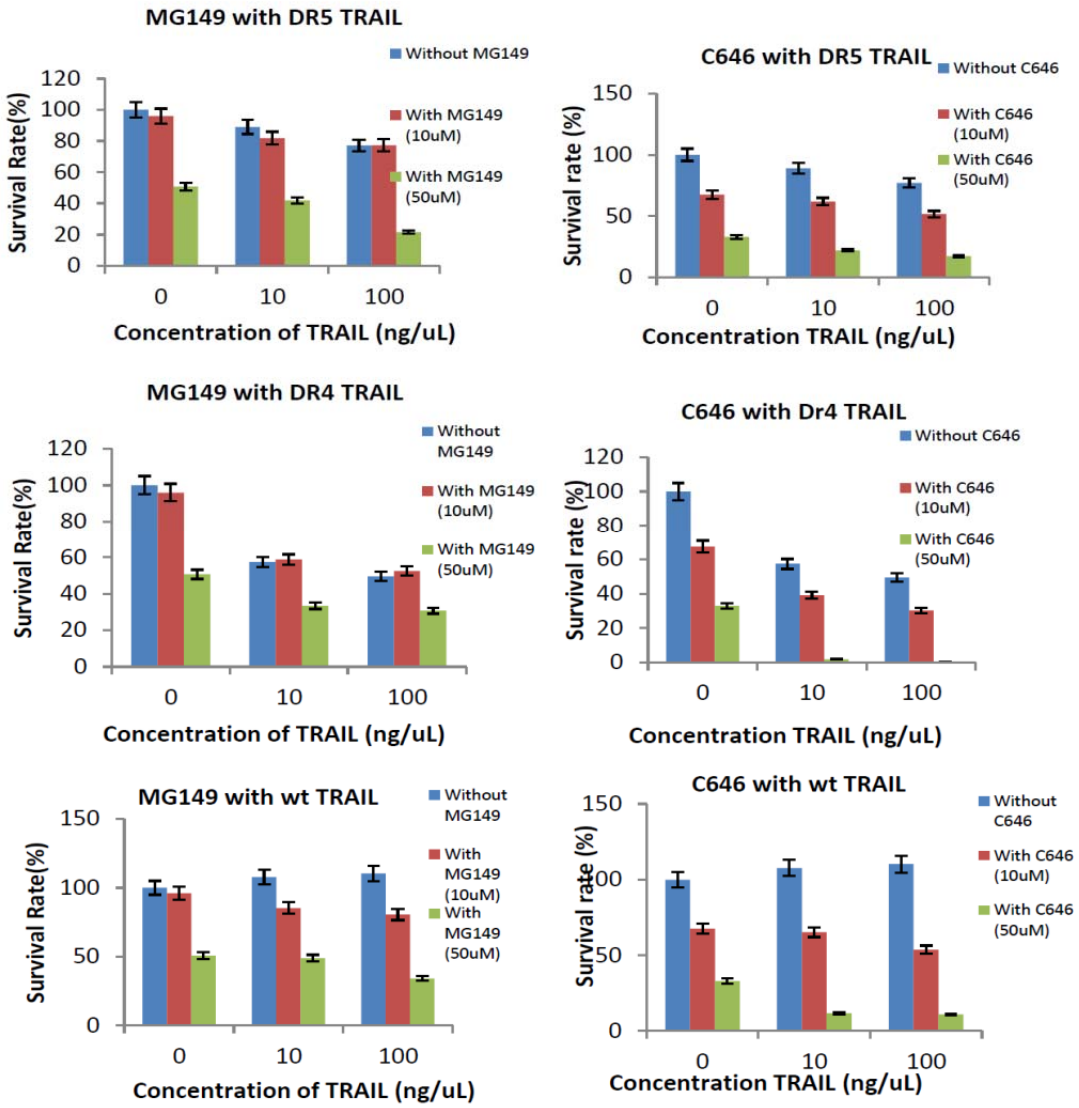

Figure 6: Simultaneous application of histone acetylase inhibitor and TRAIL enhance the efficiency of TRAIL variants in Huh-7 cell line.

Chapter 6

General discussion and future perspectives

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6

Liver fibrosis is the major problem in patients with chronic liver disease. Activation of hepatic stellate cells (HSCs) and deposition of extra cellular matrix is the major underlying mechanism of liver fibrosis, which imposes an especially hard economic and health burden on health care systems due to the cost of liver replacement therapy, the only viable option in the end stage of the disease. Therefore, the pursuit of a workable alternative therapy to reverse the fibrotic process is desired. We investigated the application of TRAIL derivatives and variants as a drug therapy to target activated HSCs. Activation of HSCs is known to be an important factor in the development of liver fibrosis. HSCs activation also occurs concurrently with the over-expression of death-inducing receptors such as Tumor Necrosis Factor Related Apoptosis-Inducing Ligand (TRAIL) receptors, including DR4 and DR5, thus rendering HSCs susceptible to the apoptotic effects of TRAIL agonists. The application of TRAIL agonists has previously been described as a potential strategy to eliminate activated HSC [1]. TRAIL agonists have been used successfully in targeting different tumors in clinical settings, proving its safety in humans. However, in order to ensure both specific targeting and efficient elimination of HSC in the fibrotic liver, further modification of TRAIL is required. It has been demonstrated that a series of human growth factors play a role in HSC proliferation in the fibrotic liver because their corresponding receptors are highly expressed in activated HSCs. Particularly, it has been shown that epidermal growth factor and platelet derived growth factor receptors expression is up- regulated on the surface of activated HSCs [2][3]. Based on this finding, we reasoned that the coupling of a single chain antibody or peptide specific to these receptors would enhance the efficiency of

TRAIL both in specific targeting and in higher loading capacity. The fusion protein produced by the coupling would be specifically targeted to and would eliminate activated HSC in the fibrotic liver. In addition, by targeting TRAIL molecules specifically to HSCs, we expected to keep them efficiently away from the hepatocytes.

In **Chapter 2** of this thesis we described the application of anti EGFR scFv–TRAIL fusion protein in eliminating activated HSCs [4]. TRAIL receptors' over expression on activated HSCs make it an ideal target for TRAIL agonists. We and others have shown that TRAIL can preferentially induce apoptosis in activated HSCs. In this chapter we present results that demonstrate the targeted elimination of activated HSCs via EGFR and simultaneous activation of the caspase pathway. Interestingly, enhanced apoptosis of activated HSCs was reduced by separately eliminating the EGFR signaling pathway through monoclonal Ab. This later finding, however, highlights the importance of coupling death signaling and survival inhibition for enhanced killing in form of an adenoviral derived fusion protein.

The application of single chain antibody for targeted delivery of TRAIL was a success, as described in **Chapter 2.** However, antibodies possess a high molecular weight, limited tissue penetration and species-specific recognition that might be disadvantageous for targeting applications in *in vivo* models and also in clinical applications. Alternatively, we imbedded GE11 and pPB peptides that recognize the PDGF receptor and EGF receptor, respectively in **Chapter 3** [5–7]. Theses peptides were embedded in form of fusion proteins with TRAIL. Whereas anti EGFR scFv-

TRAIL receptor specific peptide- TRAIL fusion proteins did not further enhance TRAIL efficiency in eliminating activated HSCs, we show that receptor expression level, proportion of surface turnover or speed of receptor internalization did not impact the efficiency of TRAIL fusion proteins in eliminating activated HSCs. In contrast, TRAIL load that was targeted to HSCs correlated with the efficiency of TRAIL fusion constructs to induce caspase dependent apoptosis in activated HSCs.

The experiments described in **Chapter 4** focus on the characterization of receptor specific TRAIL or wild type TRAIL for their role in the elimination of activated HSCs. The results presented in Chapter 4 provide evidence that more than one receptor system is involved in the recognition and signal transduction of TRAIL into activated HSCs. DR5 receptors were demonstrated to be the most frequent receptor on the surface of activated HSCs receptors. Consequently, we conclude that the selectivity of DR5 specific TRAIL is highly favorable to eliminate this type of cell. We show a substantial decrease in LX2 cell viability achieved through exposure to DR5-specific and wt TRAIL, whereas DR4-specific TRAIL was shown to have only a marginal effect on cell viability. The decrease in viability due to exposure with different TRAIL variants is concurrent with an increase in caspase 3/7 and Annexin V in HSCs. These findings suggest that the different TRAIL variants protein reduced viability in activated HSCs via caspase-associated apoptotic pathways. This finding concurs with the functional role of TRAIL in induction of death through the extrinsic caspase pathway and caspase-8 dependent activation [8].

TRAIL in non-lethal concentrations was shown to reduce production of extra cellular matrix by interfering with collagen specific HSP47 folding mechanism [9]. Our *in vitro* studies demonstrate that all TRAIL variants are capable of decreasing the expression of pro-fibrotic gene expression, such as collagen I and α-SMA production in HSCs. However, DR5 specific and wt TRAIL were proven to be the most effective proteins. We show that a reduction in collagen I and α-SMA production due to treatment with receptor specific or wt TRAILs were well correlated with a decrease in HSP 47. Together, the evidence presented argues for the successful application of the DR5 receptor-specific TRAIL variant in the targeted elimination of activated HSCs via interference with the collagen production and simultaneous induction of apoptosis via activation of the caspase pathway.

In **Chapter 5**, a combination of TRAIL variants and inhibitors for histone acetyltransferase or deacetylase (HDAC) or (HAT) was used to evaluate the potential application of different epigenetic modifications on TRAIL induced apoptosis in carcinoma cell line. Our findings indicate that simultaneous application of HDAC inhibitor SAHA could render carcinoma cell lines substantially more susceptible to different TRAIL variants. Our findings also indicate that receptor specificity of TRAIL variants did not significantly contribute to their killing effect in presence of SAHA. However, the effects of combining HAT and HDAC inhibitors in augmenting TRAIL killing effect has not yet been investigated. Our findings indicate that HAT inhibitor C646 even at low concentrations increases TRAIL-induced cytotoxicity in multiple carcinoma cell lines. In conclusion, current evidence indicates that in

conjunction with HAT inhibitors TRAIL constitutes a potent apoptotic stimulus in human carcinoma cells.

In summary, in this thesis we demonstrate that TRAIL exerts two functions in the therapy for liver fibrosis. First, a targeted form of TRAIL can be used as an inducer of caspase dependent apoptosis in activated HSCs. Second, TRAIL variants may modulate different processes such extra cellular matrix production and proliferation in activated HSCs. Thus, the dual functionality of TRAIL as a drug therapy may be successfully exploited and constitutes a new approach in the treatment of liver fibrosis.

Future Perspectives

Currently, various strategies are being investigated for their efficacy in resolving liver fibrosis, yet an ultimate solution for use in humans remains elusive. Low specificity or high toxicity of experimental drugs towards HSCs in the liver often undermines the anti-fibrotic effects *in vivo* compared with results obtained with these same compounds *in vitro.*

TRAIL application for Liver fibrosis treatment has proven its potential in therapy via *in vitro* and *in vivo* preclinical studies. However, due to the short half-life of TRAIL *in vivo,* practical use of TRAIL for chronic diseases such as liver fibrosis requires sophisticated manipulation of TRAIL in order to ensure maximum targeted uptake and persistent treatment for a long-term therapy. In this study we therefore addressed these issues by employing TRAIL in the form of a fused protein in an Ad vector for targeted elimination of activated HSCs. While the

application of adenovector ensures the long term expression of fused TRAIL protein in the liver, fusing TRAIL with targeting moieties for activated HSCs increases the chance of selective uptake by targeted cells. We also explored the efficacy of different TRAIL types in the elimination of extra cellular matrix production where different receptor specific TRAILs showed varying potential for HSC elimination and inhibition of collagen secretion mechanism by activated HSCs. These approaches open the possibility to selectively modulate the action of HSCs involved in the fibrotic process. We believe progress in vector technology and monitoring techniques could promote and enhance the idea of safely using TRAIL in treating liver fibrosis. Replacement of viral vectors by less toxic and less complex non-viral vectors that offer large carrying capacities could increase the chance of bringing the TRAIL products for treating liver fibrosis to clinic in future. Also, hopefully ever growing knowledge on molecular pathways could pave the way for application of alternative safer molecules like monoclonal antibodies for more specific targeting in eliminating activated HSCs. However, future studies using TRAIL for treatment of liver fibrosis will have to demonstrate their practical applicability in a clinical setting.

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Dutch summary

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Samenvatting

De voornaamste complicaties bij patiënten met chronische leverziekte is leverfibrose. Activering van leverstellaatcellen (Hepatic Stellate Cells, HSC) en afzetting van extracellulaire matrix is het belangrijkste onderliggende mechanisme van leverfibrose. Leverfibrose leidt tot bijzonder hoge economische- en gezondheidslasten als gevolg van de kosten van een lever transplantatie, de enige therapeutische optie in het eindstadium van de ziekte. Daarom is het streven naar een werkbare alternatieve therapie om het fibrotische proces tegen te gaan gewenst. In dit proefschrift werd de toepassing van tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) derivaten en varianten getest als een mogelijke therapie tegen geactiveerde HSCs.

Activatie van HSCs speelt een belangrijke factor in de ontwikkeling van leverfibrose. HSCs activatie en overexpressie van apoptose inducerende receptoren, zoals TRAIL receptoren, waaronder DR4 en DR5, gaan vaak samen met als gevolg dat HSCs gevoeliger worden voor de apoptotische effecten van TRAIL agonisten.

De toepassing van TRAIL-agonisten is eerder beschreven als een mogelijke strategie voor het elimineren van geactiveerde HSC [1]. TRAIL agonisten zijn met succes gebruikt bij de behandeling van verschillende tumoren in klinische setting, wat de veiligheid voor toepassing bij de mens impliceert. Echter, om zowel de specifieke en efficiënte eliminatie van HSC in de fibrotische lever te waarborgen is verdere modificatie van TRAIL gewenst.

Een aantal groeifactoren spelen een rol bij de HSC proliferatie in de fibrotische lever en groeistimulerende receptoren komen in hoge mate tot expressie in geactiveerde HSCs. Vooral epidermale groeifactor (EGF) en bloedplaatjes-afgeleide groeifactor receptoren (PDGF) komen hoog tot expressie op het oppervlak van geactiveerde HSC [2] [3].

Op basis van deze waarnemingen, denken we dat koppeling van TRAIL aan antilichamen of peptiden, gericht tegen de groeifactorreceptoren, de efficiëntie van TRAIL verhoogd zal worden, zowel in specificiteit als in hoger bindingsvermogen. Het door een moleculaire koppeling geproduceerde fusie-eiwit zou specifieker zijn en dus de geactiveerde HSC beter elimineren in de fibrotische lever. Bovendien zal door de TRAIL moleculen specifiek naar de HSCs te sturen er een minder toxisch effect van TRAIL op de gezonde hepatocyten zijn.

In hoofdstuk 2 van dit proefschrift beschrijven we de toepassing van een anti-EGFR enkele keten antilichaam, scFv, TRAIL fusie-eiwit voor de eliminatie van geactiveerde HSC [4]. De over-expressie van TRAIL receptoren op geactiveerde HSC's maken het een ideaal doelwit voor TRAIL agonisten. Wij en anderen hebben aangetoond dat TRAIL preferentieel apoptose kan induceren in geactiveerde HSCs. In dit hoofdstuk laten wij zien dat het elimineren van geactiveerde HSCs via de EGF-Receptor (EGFR) signaalroute gecombineerd met activatie van de caspase route door TRAIL tot efficiënte eliminatie van HSCs leidt. Interessant is dat de waargenomen verhoogde apoptose van geactiveerde HSC verlaagd kon worden door de EGFR signaleringsroute remmen. Deze bevinding benadrukt het belang van de combinatie van apoptose inductie en proliferatie remming.

De toepassing van scFvs voor specifieke binding van TRAIL was succesvol, zoals beschreven in hoofdstuk 2. Echter, antilichamen met hoog molecuulgewicht hebben een beperkte weefselpenetratie en zijn species-specifiek. Dit kan nadelig zijn voor toepassingen in *in vivo* modellen en in de kliniek. In plaats van anti-EGFR scFv zijn er andere liganden getest, zoals peptiden die de PDGF receptor en EGF-receptor herkennen. Deze worden beschreven in hoofdstuk 3 [5-7]. Deze peptiden werden getest in de vorm van moleculaire fusie-eiwitten met TRAIL. Anti- EGFR peptide scFv-TRAIL receptor fusie-eiwitten gaven geen verbetering van de TRAIL efficiëntie voor het elimineren van geactiveerde HSCs. Het EGFR expressie level, de mate van turnover of de internalisatie snelheid van de receptor was niet van invloed op de efficiëntie van deze TRAIL fusie-eiwitten in het elimineren geactiveerde HSC. Daarentegen correleerde de mate van binding van TRAIL aan HSCs met de efficiëntie van deze TRAIL fusie-eiwitten en het induceren van caspase-afhankelijke apoptose in geactiveerde HSCs.

De experimenten beschreven in hoofdstuk 4 zijn gericht op het bepalen van de rol van specifieke TRAIL receptoren in de eliminatie van geactiveerde HSC. De gepresenteerde resultaten leveren het bewijs dat er meer dan één receptor systeem betrokken is bij de apoptose-inductie van TRAIL in geactiveerde HSC. De DR5-receptoren bleek de meest voorkomende receptor op het oppervlak van geactiveerde HSCs te zijn. Het gebruik van DR5-selectieve TRAIL mutanten zou voordeel bieden om dit type cel te elimineren. Een aanzienlijke daling van de overleving van Lx2 HSCs werd gevonden na blootstelling aan DR5-specifiek en wt TRAIL, terwijl DR4-specifieke TRAIL slechts een marginaal effect had op de overleving van Lx2

cellen. De daling in overleving als gevolg van blootstelling met verschillende TRAIL varianten correleerde met een toename van caspase 3/7 activatie en annexine V kleuring in HSC. Deze resultaten suggereren dat de verschillende varianten van TRAIL de geactiveerde HSCs doden via caspase-geassocieerde apoptoseroutes. Deze bevinding sluit aan bij de functionele rol van TRAIL in de inductie van de celdood via de extrinsieke caspase route en caspase-8 afhankelijke activering [8].

TRAIL in niet-toxische concentraties bleek de productie van extracellulaire matrix te verminderen door te interfereren met collageen specifieke HSP47 vouwing mechanismes [9]. Onze *in vitro* studies tonen aan dat alle TRAIL varianten de expressie van pro-fibrotische genexpressie verlagen, zoals collageen I en α-SMA. Echter, DR5-specifiek en wt TRAIL bleken hierin het meest effectief. Een verlaging van collageen I en α-SMA productie door behandeling met receptor-specifiek of wt TRAIL correleerde met een afname van HSP 47. Samengevat, pleiten de gepresenteerde bevindingen voor de toepassing van de DR5-receptor specifieke variant van TRAIL voor het elimineren van geactiveerde HSC via interventie met de productie van collageen en gelijktijdige inductie van apoptose via activatie van de caspase route.

In hoofdstuk 5, werd een combinatie van TRAIL varianten met inhibitoren voor histon acetyltransferase (HAT) of Deacetylse (HDAC) gebruikt voor het evalueren van de potentiële toepassing van verschillende epigenetische veranderingen op TRAIL geïnduceerde apoptose in een carcinoma cellijn. De gecombineerde behandeling van carcinoma cellijnen met verschillende TRAIL varianten en de

HDAC remmer SAHA maakte de cellen gevoeliger voor apoptose. We vonden dat de expressie van TRAIL receptoren DR-4 en DR-5 werd geïnduceerd door verschillende HDAC remmers [10] [11] [12]. De receptor specificiteit van de TRAIL varianten droeg niet significant bij aan de toxiciteit in aanwezigheid van SAHA. Een lage concentratie van de HAT remmer C646 verhoogde de TRAIL geïnduceerde cytotoxiciteit in meerdere carcinoma cellijnen. Kortom, de combinatie van HAT-remmers en TRAIL vormt een krachtige apoptotische stimulus in menselijke carcinoom cellen.

Samenvattend, in dit proefschrift laten we zien dat TRAIL gebruikt kan worden in de therapie voor leverfibrose: Ten eerste kan een specifieke vorm van TRAIL gebruikt worden voor het selectief induceren van caspase afhankelijke apoptose in geactiveerde HSCs en niet in gezonde levercellen. Ten tweede kan TRAIL verschillende processen moduleren zoals extracellulaire matrix productie en proliferatie in geactiveerde HSCs. Aldus kan de dubbele functionaliteit van TRAIL als geneesmiddeltherapie succesvol worden benut en vormt een nieuwe benadering voor de behandeling van leverfibrose.

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Since I was a child I was so curious and tried to discover the mechanisms behind the working things. However most of the times it turned to be destroying things and understand less out of them. Still after years I feel turn of events did not quench my thirst for knowing more, yet it changed my approach on how to learn and analyse phenomenons in a more mature and sicientific manner. In this long journey of mind I am especially indebted to those whome gave me their unlimited support and patiently taught me how to proceed further after failures.

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This thesis is dedicated to my parents who have given me the opportunity of an education from the best institutions and supported me financially and spiritually throughout this program and my entire life.

I started my research with detection and characterization of genetic and antigenic variation that anchors in seasonal Influenza virus sample. Amplification of virus samples by viral culture, genomic extraction, amplification and sequencing of virus Hemagglutinin (HA), a surface antigenic protein, was a common practice. By making genomic alignment of extracted strains with seasonal vaccine strain we could detect potential antigenic shift or drift in obtained influenza samples. Data from our studies used to evaluate the efficiency of seasonal flu vaccine in preventing Influenza.

I continued my line of research by engaging a project for construction influenza cross protective vaccine that could simultaneously confer protection against more than one type of influenza virus. For this reason in a collaboration with Molecular Virology Group, UMCG we designated and produced recombinant Influenza nucleoprotein (NP), a conserved internal influenza protein, using highly efficient Baculovirus expression method for further incorporation into cross protective virosome vaccine.

Later on, I started my PhD on the topic of Development of Gene Therapy for the curing of Liver Fibrosis. Briefly I investigate the potential application of single chain antibody (scFv) –Tumor necrotic factor ligand (TRAIL) fused protein in targeted elimination of activated hepatic stellate cells (a cell type responsible for liver fibrosis). By incorporation of above protein as a payload into Adenoviral vector for further delivery and expression into liver we evaluate elimination of targeted cell type both *in-vitro* and *ex-vivo.*